THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal has been founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

A section will be devoted to short communications upon new technical methods applicable to those subjects which come within the scope of the Journal.

It is also the intention of the Editors to publish occasional "critical reviews" upon pathological subjects which may at the time be in need of critical survey. Such reviews may be of value to workers who are not in touch immediately with considered opinions upon the questions discussed.

Forwarding Papers.—Papers should be addressed to—

THE EDITORS,
British Journal of Experimental Pathology,
28, GOWER PLACE,
LONDON, W.C. 1,

or to any of the Editors personally.

Date of Publication.—The Journal is published bi-monthly, in February, April, June, August, October, and December.

Priority of Publication.—Papers accepted for publication will be printed as far as possible in the order of their receipt by the Editors, but those sent from overseas will receive priority in publication according to the date of posting instead of the date of receipt.

Conditions of Publication.—Contributors should prepare their manuscripts with the greatest attention to detail. The fact of submitting a paper for publication will be taken by the Editors as an admission by the Author of his liability to pay for all unreasonable alterations made by him in proofs, and for mistakes due to lack of clearness in the preparation of the manuscript.

Illustrations and charts will be accepted for publication, but if, in the opinion of the Editors, they are not required to increase the clarity of the argument, they must be paid for by the Authors.

References.—Authors are requested to prepare their references according to a systematic plan, namely, as given in the *Index Catalogue of the Surgeon-General's Library* at Washington. They should be numbered consecutively in the text, and collected at the end of the paper in the same order under "References." The initials, title, year, volume and page should all be included.

Proofs.—To avoid delay, proofs will not be submitted to Authors overseas unless they express a desire to see them, or unless the Editors think it necessary. Overseas Authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that error need not arise.

Reprints.—25 reprints will be supplied free to authors. Additional copies must be ordered from the Publishers *before the issue is printed*, and will be charged for according to the following scale:

		£ s. d.	£ s. d.	£ s. d.	£ s. d.	£ s. d.
Number of Additional Reprints	25	1–4 pp., 0 11 6	5–8 pp., 1 2 6	9–12 pp., 1 13 6	13–16 pp., 2 4 6	
	50	„ 0 13 9	„ 1 6 6	„ 1 19 0	„ 2 12 0	
	75	„ 0 16 0	„ 1 10 6	„ 2 4 6	„ 2 19 6	
	100	„ 0 18 3	„ 1 14 6	„ 2 10 0	„ 3 7 0	

Authors working in countries where the rates of exchange are unfavourable will be charged for reprints according to pre-war rates (25 francs = 20 shillings).

THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE OXYGEN CONSUMPTION OF NORMAL AND CANCEROUS MOUSE TISSUES *IN VITRO*.

B. R. G. RUSSELL AND W. E. GYE.

From the Laboratories of the Imperial Cancer Research Fund, London.

Received for publication May 22nd, 1920.

DESPITE the prolonged and exhaustive investigations made upon the growth of transplanted tumours, but little work has been done upon the metabolic processes which lie at the root of such growths. This is attributable in the main to the small amount of tissue to be dealt with when working with rats and mice, the two species whose tumours have lent themselves best to experimental investigation. As regards the gaseous metabolism of tumours, the only work we can cite is that of Cramer (1) and of Chisolm (2), who respectively studied the respiratory exchange of tumour-bearing rats and mice. Cramer was unable to detect any essential difference between the tumour rats and the control rats, either as regards the absolute amounts of oxygen and carbon dioxide involved or the respiratory quotient. Chisolm came to the same result after comparing tumour-bearing mice with normal mice.

Most recently Drew (3) has published observations made in this laboratory upon the rate at which normal and cancerous tissues of the mouse reduce a dilute solution of methylene blue. He has found large differences between the different tissues and lesser differences between the various tumour tissues, whilst the tumour tissues were all much less powerful in reducing the methylene blue solution. We shall have occasion to refer to these findings more particularly later.

In attempting to assess the oxygen consumption of tumour tissue one is severely handicapped by several factors, the more serious being the restriction of choice of animal, and the defective circulation in tumours, with its concomitant load of dead tissue taking next to no part in the gaseous exchange but upsetting the calculation of quantities severely. The method so successfully applied by Barcroft and his co-workers in estimating the oxygen consumption of various organs from the different content of oxygen in afferent and efferent blood is not available. The blood-vessels of mice and rats are too small and delicate to allow of this manipulation. The successful implantation of a suitable tumour into the kidney of such an animal as the rabbit might supply the necessary conditions for an analysis on those lines, but no such tumour was available. Under these circumstances we were impelled to conduct our investigations in a less ideal condition, and to test the oxygen consumption of the normal and cancerous tissue *in vitro*.

It has long been known that the tissues of an animal are capable of absorbing oxygen and of giving off carbon dioxide for a considerable time after the death of the animal. More recently Battelli and Stern (4), in a series of papers, have published their findings on post-mortem respiration. In general their method of procedure was to suspend a fine emulsion of the tissue in blood and expose it to an atmosphere of oxygen at 38° C. The readings they obtained were very high; pigeon muscle in ox blood absorbed oxygen at the rate of 66 c.c. per kilogram minute; dog's liver in an emulsion of dog's red blood cells absorbed 60 c.c. per kilogram minute. Harden and Maclean (5), in an extensive series of experiments, were unable to confirm these high readings, and the figures they obtained showed an oxygen consumption which was only a tenth or a twelfth of what Battelli and Stern obtained. An emulsion of dog's liver suspended in ox blood, for example, was found to consume 3·8 to 6·6 c.c. per kilogram minute, figures differing radically from those of Battelli and Stern.

Before we came to the estimation of the oxygen consumed by different transplanted mouse tumours, it was necessary to obtain figures for various normal organs of the mouse, as it was only to be expected that the mouse with its very active metabolism would yield figures very different from those found by Harden and Maclean for larger animals. The only observation made on mouse tissue was that of Usui (6), who estimated the oxygen consumption of the left lobe of the liver in a 10 per cent. suspension of ox blood corpuscles and found figures varying from 4 to 9 c.c. per kilogram minute. Usui differed from his predecessors in not using an emulsion of tissue but in dealing with the lobe of liver in its entirety, but this obviously had the drawback of the central portion not receiving an adequate supply of oxygen.

The method employed in making the present series of determinations has been as follows:—With the exception of two experiments done with mouse blood, rabbit blood has been used throughout as a source of oxygen. This has

been obtained from an ear vein after thorough cleansing with ether; in this way it is an easy matter to obtain 30 c.c. or more of defibrinated blood which is often sterile, and never contains more than a few organisms which do not disturb an experiment of two or three hours' duration. Absolute sterility of the blood need not be aimed at, since the mouse tissues to be investigated usually contain a few organisms. The blood is oxygenated by shaking or rotating for five minutes in a flask. The mouse is then killed and the tissue to be tested removed and rapidly emulsified with a pair of curved scissors. With tumours, care has been taken to remove all naked-eye necrotic material. A weighed quantity of this is added to a measured quantity of the rabbit's blood in a cylindrical glass vessel of 28 mm. diameter provided with a stirring rod and covered with a layer of liquid paraffin. The quantities commonly employed have been half a gramme of tissue emulsion in 12 c.c. of blood, these quantities being varied either way with a view to attain the consumption of half the available oxygen after two or three hours' incubation. The mixture is then incubated in a water-bath at 37·5° C. A control tube containing 10 c.c. of blood without any added tissue but otherwise similarly equipped is also incubated. This tube is necessary to ascertain the rate of auto-oxidation, which has been found to vary considerably. Ten minutes' incubation is allowed for equilibrium to be established, and the first sample of blood is then withdrawn with a 1 c.c. pipette containing a little warm paraffin. The sample is next analysed in Barcroft's blood-gas apparatus, and the amount of oxygen necessary to complete saturation is estimated. This is repeated after further incubation at hourly intervals for the blood and tissue tube, but for the control tube, on account of the small differences noted, a second sample has usually been taken only after two or three hours' incubation. In all the experiments the time elapsing between the death of the mouse and the taking of the first blood sample has been between twenty and twenty-five minutes.

The course of an experiment will be seen in the following protocol where the oxygen absorption of liver emulsion is given in detail.

14/4/20.—Rabit bled at 11.15 a.m.; animal last bled twenty-two days previously. Mouse killed at 11.20 a.m., and 0·63 grm. of liver emulsion added to 13 c.c. of defibrinated rabbit's blood. As control 9 c.c. of blood was taken. Incubation started at 11.34 a.m. in water-bath at 37·5° C.

Blood with emulsion of liver:

Barcroft apparatus $\frac{3}{4}$ (K = 3·36).	Temp., 14° C.	Bar. Pr., 750 mm.		
Time	11.45 a.m.	12.45 p.m.	1.45 p.m.	2.45 p.m.
Oxygen absorption in mm.	4·5	14·5	26·5	30

Control blood:

Barcroft apparatus $\frac{1}{2}$ (K = 3·17).	Temp., 14° C.	Bar. Pr., 750 mm.
Time	11.50 a.m.	2.50 p.m.
Oxygen absorption in m.m.	3	5

Re-calculation of the above figures after allowance for auto-consumption gives during the first hour an oxygen consumption of 9·2 c.c. per kilogram minute; 10·0 c.c. for the second hour; and 2·3 c.c. for the third hour. The auto-consumption of the blood was exceptionally low in this experiment; more usually the figure obtained is 0·0060 volumes per cent. per minute. The highest figure found was 0·0160, but the rabbit had been bled only ten days before. There was no evidence pointing to the rate of auto-consumption affecting that of the tissue tested either way.

In Table I the rate of oxygen consumption is summarised for the different normal tissues tested, the figures being given in c.c. per kilogram minute.

TABLE I.

Organ.	First hour.	Second hour.	Third hour.	Amount of tissue in grms.
Kidney	21·5	20·1	16·1	0·42
Ditto	23·9	19·5	5·8	0·28
Ditto	18·5	15·9	—	0·44
Average	21·3	18·5	—	—
Liver	12·9	9·4	4·9	0·35
Ditto	9·2	10·0	2·3	0·63
Average	11·0	9·7	—	—
Testis	8·4	6·7	—	0·60
Ditto	4·7	4·3	6·5	0·35
Ditto	4·3	—	—	0·53
Average	5·8	5·5	—	—
Embryo	4·0	3·0	3·0	0·55
Ditto	3·0	—	—	0·66
Mamma 4 days lactating	9·8	7·0	4·4	0·56
Mamma 2 days lactating	9·3	—	—	0·42
Mamma—full term	9·1	—	—	0·33
Mamma about 3 days before full term	5·1	4·7	3·4	0·66
Mamma about 6 days before term	7·2	5·6	1·3	0·30

The mice whose tissues have been tested have all been fed in the ordinary way—that is they were living with a surplus of food at their disposal. No attempt was made to analyse the oxygen consumption of the tissues of fasting animals, although deprivation of food might very possibly affect the figures for such organs as kidney and liver. The oxygen consumption found for the various tissues is seen to differ materially, whilst the value found for any one tissue in

separate experiments has been fairly close except in the case of testicular tissue, which has behaved rather erratically. The second hour readings show a distinct drop in almost all cases, whilst this drop is very marked in the third hour. In later experiments a third hour reading has more usually been omitted on account of its irregularity. Two further experiments were done on mouse kidney suspended in defibrinated mouse blood, and the values found were a third higher than in rabbit blood. The high rate of auto-oxidation of mouse blood, about six times that of rabbit, made it unsuitable for testing the more slowly oxidising tissues.

The rate of consumption by kidney emulsion in mouse blood, about 30 c.c. per kilogram minute, does not approach what might be expected for this organ under physiological conditions. Barcroft and Straub (7) in their work on secretion of urine found the kidney of the cat and rabbit usually consumes 70 to 100 c.c. per kilogram minute, and the figure for mouse kidney might well be expected to be higher. The comparatively low readings obtained when using an emulsion indicate that in the process of mincing the cells are damaged to a considerable extent. In this connection reference may be made to the deleterious influence which mechanical injury has upon the oxidative transformation of lactic acid in excised amphibian muscle as described by Fletcher and Hopkins (8). They emphasise rightly the necessity of drawing a distinction between the study of chemical processes in minced tissues and intact living tissues.

The findings for mammary tissue require special consideration, as here one is not dealing with an organ whose composition is even relatively constant, but varies considerably with the functional condition of the gland and the state of nutrition of the animal. To ascertain the degree of this variant, a large lobe of mamma was preserved for microscopic examination in each experiment, and the conditions found in each case were as follows.

The four-day lactating mamma showed about a third of the section occupied by fatty tissue. The parenchyma consisted mainly of somewhat dilated acini lined by a layer of cubical epithelium. The two-day lactating mamma contained very little fatty tissue, and the field showed a larger amount of glandular epithelium, whilst the acini were not so dilated. The mamma of the mouse taken at full term showed a large amount of fat occupying rather more than half the section, whilst the acini were more contracted. The mammæ from the eighteenth day of pregnancy and from the fifteenth consisted of equal parts of fatty tissue and parenchyma; the acini were very dilated, rendering it difficult to estimate the amount of epithelium present, but it was approximately equal to that in the full-term mamma. Comparison of these histological findings with the respective rate of oxygen consumption found *in vitro* fails to establish a parallel in the sense of a direct relationship between the amount of oxygen consumed and the amount of parenchyma present. So far as any conclusion can be drawn from the limited number of observations, it is that

the oxygen consumed is parallel to the functional activity of the gland. In the first three cases milk was being secreted in visible quantities, and the rate of oxygen consumption was appreciably higher than it was in the other cases where no milk was seen. The large differences found in the oxygen consumption of kidney, liver and embryo tissues make it rather difficult to believe that one is dealing with the true basal metabolism of these tissues. A part of the difference might, in the case of embryo tissue, be attributed to its high water content, 85 per cent. for embryo tissue, against 77 for kidney and 73 for liver; but allowance for this factor still leaves an astonishing discrepancy. In the embryo the quantity of tissue functioning in the sense of doing work is small, and this again suggests that the much higher reading for kidney and liver tissues is due to a partial continuance of function for an hour or two under the conditions of the experiment. Still, as Warburg (9) has pointed out, there may be a high oxygen consumption with minimal expenditure of energy in performing work, as in the case of the isolated central nervous system of the frog, or in the nucleated red blood-cells of birds. He suggests (on page 259) that mechanical work may be necessary to retain the complicated cell structures inviolate.

The experiments on tumours have been carried out in the same way as for normal tissues. In preparing an emulsion of tumour tissue great care has been taken to remove necrotic areas, but histological control has shown the impossibility of eliminating entirely the dead tissue, and at best one can only aim at reducing it to a minimum which will be fairly constant in successive experiments. Seven transplanted mouse tumours have been tested, including six carcinomata of different rates of growth and a rapidly growing sarcoma, and the figures obtained are presented in tabular form in Table II.

TABLE II.

Tumour.	First hour.	Second hour.	Third hour.	Amount of tissue in grammes.
Carcinoma 206	9·7	5·7	—	0·44
Ditto	5·5	2·6	—	0·65
Sarcoma 37	5·5	6·2	—	0·56
Ditto	9·3	7·4	—	0·60
Ditto	5·4	4·9	0·8	0·57
Average	6·7	6·2	—	—
Carcinoma 63	5·7	4·9	—	0·60
Ditto	6·7	0·0	3·8	0·48
Ditto	6·1	—	—	0·46
Average	6·2	—	—	—
Carcinoma 199	5·3	3·5	—	0·67
Ditto	4·0	—	—	0·69

Tumour.	First hour.	Second hour.	Third hour.	Amount of tissue in grammes.
Carcinoma 113	3·8	4·4	3·4	0·63
Ditto	4·8	0·6	3·2	0·60
Carcinoma 72	5·4	1·2	—	0·48
Ditto	6·4	1·4	—	0·60
Carcinoma 155	3·5	1·8	—	0·80
Ditto	8·8	2·1	3·0	0·44

From this table it will be seen that there is, in general, about the same drop in the rate for the second hour as was previously noted for the normal tissues. The consumption during the first hour further shows considerable differences for the same tumour strain. These wide differences in the same tumour strain we believe to be actual, and not accidental or attributable to errors of technique. Sarcoma 37, a rapidly growing spindle-celled tumour, for example, shows a wide variation between 5·4 c.c. and 9·3. The most consistent readings are those with carcinoma 63, a rapidly growing carcinoma which has been growing very uniformly for several years. Carcinoma 206 can at times grow with extreme rapidity, but it behaves very erratically. The high reading of 9·7 c.c. was obtained from such a rapidly growing specimen.

The very discrepant readings obtained with carcinoma 155 seem also to coincide with a differing rate of growth. This is the slowest growing tumour tested, but the specimen which gave the high reading grew exceptionally fast, although still very slowly when compared with strain 63.

In Fig. 1 the rates of oxygen consumption have been plotted both for the normal and neoplastic tissues, the average figures obtained for the first hour being expressed in terms of 5 mm. height, equalling 1 c.c. per kilogram minute. When graphically represented in this way, it can be readily seen how slight are the variations in the rates for neoplastic tissue. If a horizontal be drawn through the top of the column representing the mammæ of pregnant mice, it will be seen how the tumours group themselves about this line. Whether the tissue histogenetically related to a tumour determines approximately the rate of oxygen consumption of the latter could only be settled by comparison with transplanted tumours of other organs such as kidney or liver, material which unfortunately is not at present available.

It will be noted that the other rapidly growing tissue tested, the embryonic tissue, has a very low oxygen consumption. There is no reason to assume that this complex of tissues is more severely damaged by cutting than the other tissues, but it seems more reasonable to suppose that it works more economically than the tumour tissue.

The five columns in the figure representing carcinoma 206, sarcoma 37, and the carcinomata 63, 199 and 113, show a regular diminution. This diminution is so far consistent in that it agrees directly with the speed of

naked-eye growth of the tumour strains in question. The sarcoma, as already stated, is a rapidly growing spindle-cell tumour of mammary origin, whilst the four epithelial tumours are all alveolar mammary carcinomata of different rates of growth. In all the histological structure shows an extreme degree of loss of histological differentiation. The most slowly growing tumour of this group, carcinoma 113, differs from the other three in that its cells show a unique aptitude for storing glycogen, a feature present in the primary growth and now observed in the transplanted tumour for many years.

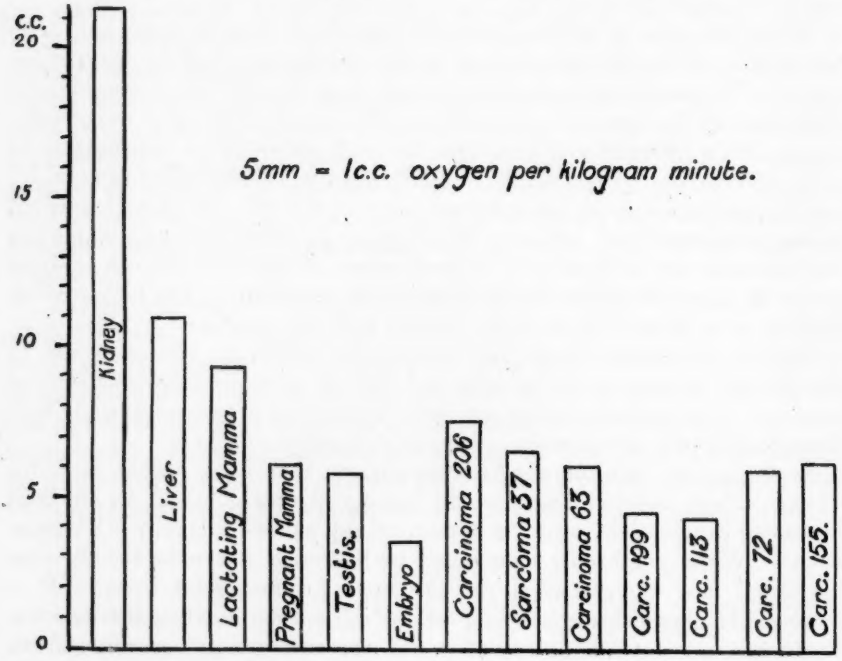


FIG. 1.—Diagrammatic representation of average rate of oxygen consumption for the first hour.

The two carcinoma strains 72 and 155 differ in two respects from the previous group. They grow very much more slowly, and show a higher degree of histological differentiation, the acinous structure of the parent mammary tissue being retained in a varying degree of perfection.

The remarkable differences which Drew found between normal tissues on the one hand, and neoplastic tissues on the other, do not appear in anything approaching the same degree in our experiments. This is attributable to the different conditions under which the tissues were tested by Drew. Whereas all our estimations have been carried out on tissues with a readily available supply of oxygen, in Drew's experiments the cells were virtually existing

under anærobic conditions, and the oxygen which they used had to be split off from water, leaving the hydrogen free to reduce methylene blue.

So far as the limited differences which we have found in the rate of oxygen consumption by various transplanted tumours allows us, the following conclusions will be tentatively drawn :

That the rate of oxygen consumption rises with increasing speed of growth, and further that more oxygen is used by tumours which are more highly differentiated histologically.

REFERENCES.

1. Cramer, W.—"The Gaseous Metabolism in Rats inoculated with Malignant New Growth," *Third Sci. Rep. Imp. Cancer Res. Fund*, London, 1908, p. 427.
2. Chisolm, R. A.—"The Respiratory Exchange of Mice bearing Transplanted Carcinoma," *J. Pathol. and Bacteriol.*, 1910, **15**, 192.
3. Drew, A. H.—"The Comparative Oxygen Avidity of Normal and Malignant Cells Measured by their Reducing Powers on Methylene Blue," *Brit. J. Exper. Pathol.*, 1920, **1**, 115.
4. Battelli, F., and Stern, L.—"Recherches sur la respiration élémentaire des tissus," *J. de physiol. et pathol. gén.*, 1907, **9**, 1, 34 and 128; *Compt. rend. Soc. de biol.*, 1907, **62**, 296, 386, 531, 596, 832, 958 and 1110.
5. Harden, A., and Maclean, H.—"The Oxidation of Isolated Animal Tissues," *J. Physiol.*, 1911, **42**, 34.
6. Usui, R.—"Ueber die Messung von Gewebsoxydation in Vitro," *Arch. f. d. ges. Physiol.*, 1912, **147**, 100.
7. Barcroft, J., and Straub, H.—"Secretion of Urine," *J. Physiol.*, 1910, **41**, 145.
8. Fletcher, W. M., and Hopkins, F. G.—"Lactic Acid in Amphibian Muscle," *J. Physiol.*, 1906, **35**, 247.
9. Warburg, O.—"Beiträge zur Physiologie der Zelle insbesondere über die Oxydationsgeschwindigkeit in Zellen," *Ergeb. d. Physiol.*, 1914, **14**, 253.

ON GLANDULAR ADIPOSE TISSUE, AND ITS RELATION TO OTHER ENDOCRINE ORGANS AND TO THE VITAMINE PROBLEM.

W. CRAMER.

From the Laboratories of the Imperial Cancer Research Fund, London.

Received for publication May 26th, 1920.

ON THE OCCURRENCE AND STRUCTURE OF GLANDULAR ADIPOSE TISSUE.

THE adipose tissue in most species of animals, including man, is apparently of a uniform character in different sites of the body to the naked eye and when examined by the ordinary histological methods, namely in frozen sections stained with sudan, or in paraffin sections stained with osmic acid either directly or after previous bichromate fixation. It will be pointed out presently that this uniform appearance is only superficial and that sometimes distinct differences can be noticed. In some species of animals, however, such as the white rat and the tame mouse, for instance, two distinct types of adipose tissue can always be recognised even by the naked eye. In addition to the ordinary adipose tissue with which everybody is familiar there occurs in these species a second gland-like type of tissue, the cells of which are filled with globules giving all the typical histo-chemical reactions for fat.

It can be seen in various sites in well-defined reddish-brown masses which contrast strongly with the yellowish-white fat of the ordinary adipose tissue. The three sites where it is most abundant are—

(1) On the back between the scapulæ with extensions between the muscles. The tissue lies here in two oval, definitely circumscribed, gland-like masses, each about 1 cm. long. Smaller masses may be found also in the axilla and in the neck (Fig. 1, Plate I).

(2) In the thorax in front of the vertebral column. It appears there as a narrow brown band stretching through the thorax along the thoracic aorta (Fig. 3, Plate I).

(3) In the abdomen between the kidneys and the aorta. It lies on either side of the renal vessels, and extends both above the kidney, enclosing the adrenal, and below the kidney, enclosing (in female animals) the ovary (Fig. 4, Plate II).

Our observations have been made mainly on the perirenal cholesterin fat

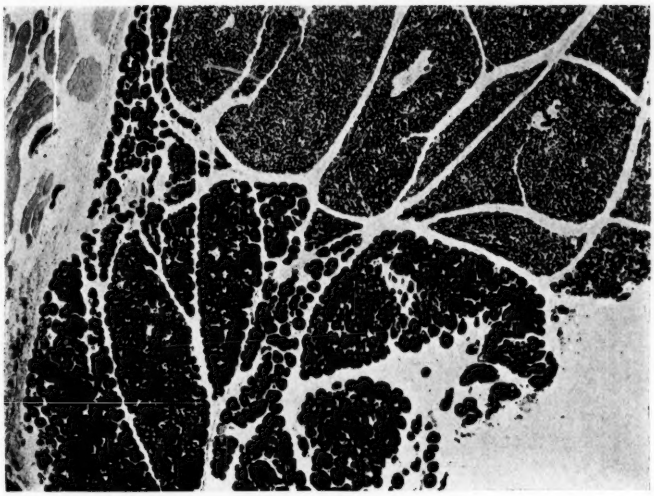


FIG. 1.

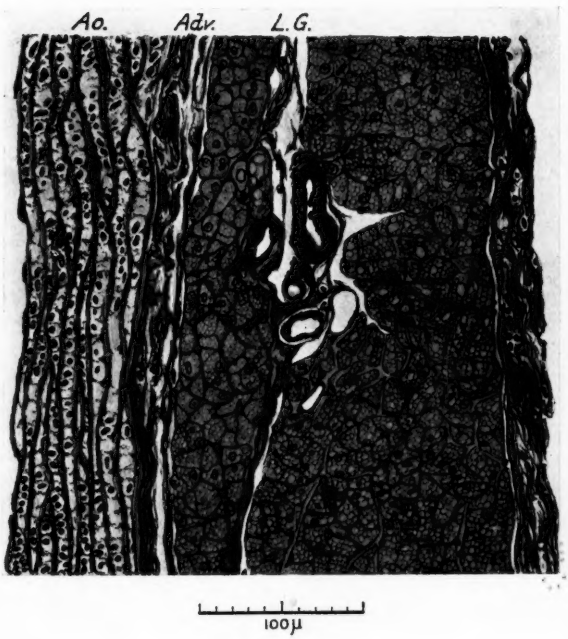


FIG. 3.

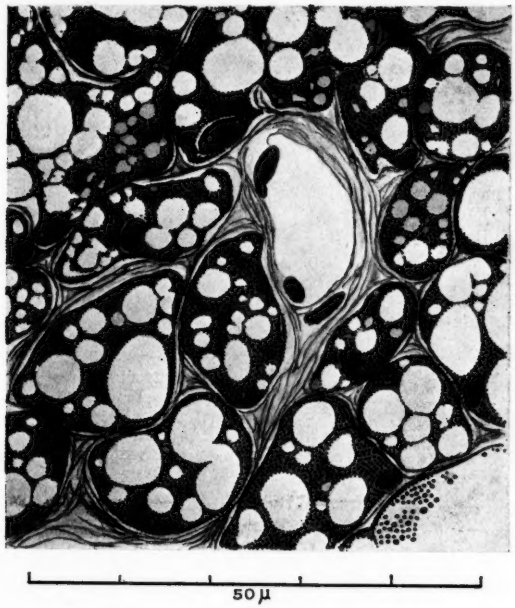


FIG. 4.

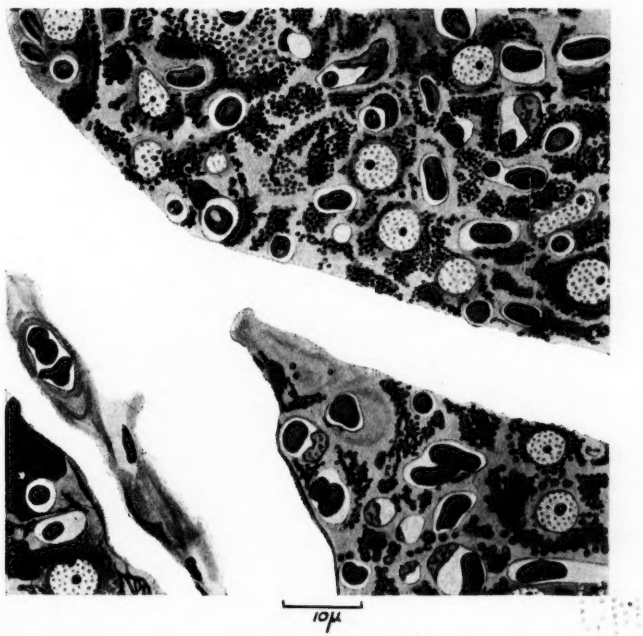


FIG. 5.

Plate II. Cramer. On Glandular Adipose Tissue.

tissue and the following descriptions refer more particularly to it. Frozen sections show that the cells of this tissue contain globules which stain deeply with sudan and osmic acid. In some parts of this tissue the cells may contain only one large globule, which squeezes the nucleus to one side as in ordinary adipose tissue; in other parts, however, the tissue is made up of cells containing numerous small globules which are grouped around a central nucleus. These cells are polygonal, not round, and appear to be richer in protoplasm than the round cells of the type found in ordinary adipose tissue. This is especially well shown in paraffin sections fixed in formol or in Zenker's mixture, in which all the fat and lipoids have been dissolved out but the protoplasm has remained. In such sections the reticulated appearance of the polygonal cells with a central nucleus forms a striking contrast with the empty, oval cells of ordinary fat with the nucleus pressed against one side. The cells are arranged in distinct lobules which are richly vascularised. In the rat, in fact, this gland-like adipose tissue resembles structurally the hibernating gland as described by Carlier (1) in the hedgehog. The clearest histological picture of this tissue with the globules *in sitú* is obtained by treating the tissue with osmic acid after a previous application of potassium bichromate. The method used by us has been that recommended by Schridde for the demonstration of Altmann's granules, viz. nxing the tissue in formol-Müller's fluid for one day and, after transferring it first to Müller's fluid for two days, then to osmic acid 2 per cent. for two days, embedding it in paraffin. If such sections are examined without further staining one obtains the picture illustrated in Fig. 1. When examined in polarised light under crossed Nicols in frozen sections many of these globules show very distinct double refraction, which disappears on gently warming the slide and reappears on cooling. This anisotropic fatty material is mixed with isotropic fatty material—in other words, the globules contain a mixture of true fats and lipoids. From its histo-chemical reactions it appears that the anisotropic material consists of a mixture of several lipoid substances, since the bichromate-osmic acid treatment does not stain all the lipoids present in the tissue. We know from previous observations (2) that this histo-chemical reaction enables us to differentiate between different lipoids. It gives positive results with true fats and with some lipoids, but negative results with other lipoids.

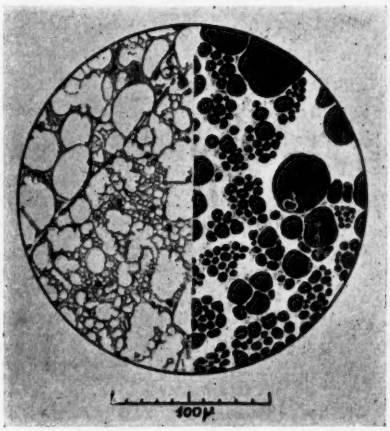


FIG 2.—(For description *vide* p. 196.)

Of the lipoids present in these globules one group can be readily identified as belonging to the cholesterol group. For if some of this tissue is dried, suspended in warm chloroform and the solution tested by the Liebermann-Burkhardt test by the addition of 10–15 drops of acetic anhydride and one or two drops of concentrated sulphuric acid, the typical greenish-blue colour develops after an interval of a few minutes.

It must be emphasised that the mere presence of anisotropic lipoid is not one of the features which distinguish this glandular type of adipose tissue from ordinary adipose tissue. The latter, too, contains anisotropic lipoids, of which one can be identified as belonging to the cholesterin group by means of the Liebermann-Burkhardt test. Nor is it possible to predict from the morphological appearance of a fat cell whether anisotropic material is present or not. It occurs not only in the polygonal cells with a central nucleus and several globules but also in the large round cells with only one large globule, although in the latter there is a preponderance of isotropic true fat. Whether all the different lipoids present in the glandular type of adipose tissue are also present in ordinary adipose tissue cannot at present be decided.

In the guinea-pig, the rabbit and in man, the fat in the immediate neighbourhood of the kidney has been studied in the same way. To the naked eye the adipose tissue in this region does not offer any obvious differences from that of ordinary adipose tissue. In man the so-called perinephric fat within the perinephric fascia sometimes feels firmer and more lobulated than the ordinary fat, but the difference is slight and is not always noticeable. Paraffin sections prepared by Schridde's method, or frozen sections stained with sudan, may show, as a rule, no difference so far as the purely morphological picture is concerned from ordinary adipose tissue, *i. e.* the cells contain one large fat-globule. Sometimes, however, one may find the same appearance as that found constantly in the rat and the mouse—polygonal cells filled with a number of small fat-globules grouped around a central nucleus. Fig 2, which is drawn from a Zenker and a Schridde preparation of human perinephric fat from a child, illustrates this point. The same appearance has been found occasionally in adults.

Although in animals other than the tame mouse and the white rat our observations have been made mainly on the perinephric tissue, the glandular adipose tissue is not restricted to that region. In the guinea-pig, for instance, it occurs in considerable masses in the neck on either side of the trachea. surrounding the glandular structures situated there (thyroid and lymphoid nodules). Its distribution in the human subject will be dealt with below.

The proportion between double refracting material and ordinary isotropic fat is very variable. The perirenal adipose tissue of the mouse and the rat is rich in anisotropic material in proportion to the amount isotropic fat present, In the guinea-pig, rabbit, and man, the isotropic material preponderated in most of the specimens of perinephric fat which have been examined.

The deposition in the form of numerous small globules is probably partly due to the fact that the cells are rich in protoplasm. But this is not the only determining factor. As long as the amount of anisotropic material is relatively large in proportion to the amount of ordinary fat, the fatty material arranges itself in the cells of this tissue in numerous small globules around a central nucleus. When the relative amount of true isotropic fat increases, as it does in well-nourished animals, these small granules as they increase in size tend to push the nucleus to one side of the cell and to coalesce into one large drop. The difference between the perirenal glandular adipose tissue of the rat and the mouse on the one hand, and of most other species on the other, is that in these latter ordinary true fat may be deposited to a variable extent in the cells of the glandular adipose tissue, so that this tissue assumes the external appearances of ordinary adipose tissue. In the mouse and the rat, on the other hand, this process does not take place to the same degree, and the tissue retains to a greater extent its distinctive appearance and structure. It follows, too, that the morphological differences—the peripheral or central position of the nucleus, the round or polygonal shape of the cells, the apparent poorness or richness in protoplasm—are not of essential significance, but probably only secondary effects resulting from changes in the size and the number of "fat"-globules present.

In the tame mouse and the white rat the glandular adipose tissue between the scapulæ and in the thorax differs somewhat from that around the kidney. Macroscopically the interscapular tissue is firmer, more definitely circumscribed, and thus conveys more the appearance of an organ or a gland. Microscopically it is more definitely arranged in lobules in which the pleoprotoplasmic, polygonal, polyglobular cells with a central nucleus predominate, especially in the thoracic fat, while under normal conditions the perinephric tissue is composed mainly of the round monoglobular cells with a peripheral nucleus. On examination in polarised light the phenomenon of double refraction is much less marked in the case of the thoracic and scapular tissue. This may be due to differences in the absolute amounts of cholesterin compounds and other double refracting lipoids present in the different sites. But it may merely be a mechanical effect resulting from the fact that in the thoracic and scapular tissue the lipoid globules are smaller and more finely dispersed than in the perirenal tissue. This latter explanation is suggested by the fact that the monoglobular round cells of the scapular fat are as strikingly double refracting as those of the perirenal tissue, while a faint double refraction is shown by the multiglobular cells. It is at present not possible to say whether these differences between the tissues in the different sites and between the two types of cells described have a functional significance.

The readiness with which the glandular adipose tissue can be recognised in the rat and mouse renders it easy to demonstate that this tissue is also functionally different from ordinary adipose tissue. For in conditions such as

fasting or a restricted diet, in which the ordinary adipose tissue is greatly diminished, the glandular adipose tissue persists and shows no obvious diminution. Conversely, in very fat animals the glandular adipose tissue is not obviously increased, although it is lighter in colour and becomes surrounded by ordinary adipose tissue. Microscopically the tissue of well-nourished animals shows a mixture of cells of the monoglobular and of the polyglobular type. When the diet is restricted the monoglobular type of cell becomes transformed into the polyglobular type, so that eventually the tissue is composed of cells containing many small globules, and these cells retain their load of lipoids and fats even when the ordinary adipose tissue has entirely disappeared. The same observation has been made by Hammar (3), in his important study of the histogenesis of this special fat, which he called the "brown fat." His conclusions will be discussed in greater detail below. The difference in the behaviour of these two different types of fat under different nutritional conditions is so remarkable that some of the figures obtained by Hammar may be quoted here:

	White fat.	Brown fat.
Animal on restricted diet for 3 weeks	0·53 per cent. of body-weight.	0·60 per cent. of body-weight.
Animal on normal diet	4·5 „ „	0·64 „ „
	5·0 „ „	0·91 „ „
Animal made obese by overfeeding for 3 months	7·0 „ „	1·51 „ „

In other species where the glandular adipose tissue may become infiltrated to a marked degree with ordinary true fat conditions are obviously more complex, and further investigations are necessary to determine the behaviour of this tissue under different nutritional conditions.

ON THE HISTOGENESIS OF THE GLANDULAR ADIPOSE TISSUE.

If it is true that the glandular adipose tissue is a functionally distinct type of adipose tissue one would expect to find that it should also be histogenetically distinct from ordinary adipose tissue. That expectation is fulfilled. A study of the extensive literature on the development of adipose tissue shows that the embryos of all mammalian species that have been studied (rabbit, guinea-pig, cat, dog, calf, pig, squirrel, rat, mouse, marmot, hedgehog, man) show a form of adipose tissue the cells of which are arranged in definite lobules, are relatively rich in protoplasm, and contain a number of fat-granules grouped round a central nucleus. This "gland-like" fat tissue, as it has been called, may persist for some time after birth, and in the case of infants has been described and figured by Batty Shaw (4) as occurring in the subpleural tissue, in the axilla and around the kidneys. In the human subject the accumulation of this tissue which is situated in the neck has attained the dignity of a separate anatomical organ, and has been described by Hatai (5), and later, in greater detail, by Bonnot (6), under the name "the interscapular gland."

According to Bonnot its situation is on the shoulder and side of the neck between the upper border of the scapula and the clavicle, running forward, towards the trachea, enclosing the large blood-vessels, while a large process extends upwards to the mastoid process. According to the same observer. "It is pink in colour and lobulated, presenting much the appearance of a fresh pancreas," and being macroscopically recognisable even in the adult subject. The tissue undergoes hypertrophy under conditions of deficient thyroid secretion. In human cretins circumscribed swellings of "fatty" tissue have frequently been noticed to occur in the axilla and in the neck. This fact was first observed by T. B. Curling (7), who described it as "fat of a loose lobulas structure," in 1850, and his account was confirmed by Hilton Fagge (8) in 1871. Recently Shattock (9) has pointed out that this fat is not composed of ordinary adipose tissue, but is a hypertrophy of the gland-like lobulated adipose tissue which occurs normally in this region in infants. This hypertrophy suggests the existence of a functional relationship between the activity of this glandular adipose tissue and that of the thyroid. There is also evidence of such a relationship between the adrenal glands and this tissue. This will be discussed in greater detail in the last part of this paper.

In most species the characteristic histological appearance of the cells of this tissue disappears soon after birth, and the majority of authors state that it is transformed into the ordinary type of adipose tissue. In some species, however (white rat, tame mouse, and the hibernating animals), it retains its characteristic structure.

A good deal of discussion has centred around the morphological significance of this embryonic gland-like fat tissue, and the only point on which all authors are agreed is the assumption, now disproved by the observations recorded in this paper, that the fat contained therein consists of ordinary true fat and is essentially the same as that of fully-developed adipose tissue. It has been held by some authors that the adipose tissue of the adult animal is a tissue *sui generis*, and that all the cells of the adipose tissue of the adult animal are derived from this gland-like accumulation of fat tissue found in embryos, which has therefore been called "the primary fat organ." Another school of morphologists holds that adipose tissue is formed simply by the deposition of fat in connective-tissue cells, and that the peculiar appearance of adipose tissue in the embryo is due merely to the deposition of fat in embryonic connective-tissue cells. An intermediate position is taken up by Hammar (3), who has been able to distinguish in the embryo two distinct types of adipose tissue formation. In the one type the fat is deposited in ordinary connective-tissue cells which show no special arrangement in lobules; this he calls "secondary adipose tissue formation." The other type, which he calls "primary adipose tissue formation," proceeds in special groups of connective-tissue cells which show a distinct arrangement in lobules even before fat-globules become deposited in them. The cells, which are at first small, increase in volume

owing to an increase in protoplasm and become polygonal. Fat is deposited in them in the form of small globules. We have, then, the embryonic gland-like fat tissue called by some authors the "primary fat organ." The fat-globules increase in size and eventually coalesce to form one large fat-globule, which gives the impression that this tissue is gradually being transformed into the ordinary type of adipose tissue. In the white rat, however, the process of "primary adipose tissue" formation remains in the embryonic stage, and we then get the "brown fat."

The glandular adipose tissue with which this paper deals is derived from the special groups of connective-tissue cells which exhibit the process called the "primary adipose tissue formation" by Hammar. In other words, it is histogenetically distinct from ordinary adipose tissue.

ON THE FUNCTIONAL SIGNIFICANCE OF GLANDULAR ADIPOSE TISSUE IN RELATION TO VITAMINES.

When rats or mice are fed on a diet of purified casein, starch, salt mixture, and olive oil, which is completely free from all vitamines, this glandular adipose tissue loses its fat and lipoid content, and in the terminal stages is completely free from either fat or lipoid. It has then the appearance of a glandular organ, as depicted in Fig. 5 (Plate II). The cells are large, rich in protoplasm, and filled with well-stained granular mitochondria. They contain on material which stains with either sudan or osmic acid vapour or osmic acid before or after bichromate fixation. Nor do they contain cell inclusions which are anisotropic or which are soluble in the ordinary fat-solvents, since paraffin sections of formol or Zenker-fixed material do not show any vacuoles in the cells. Frequently this tissue, especially the scapular tissue, is so deeply congested that it is purple in appearance and almost looks like a blood-clot. Sections of such a specimen reveal the very vascular nature of this tissue, for every cell can then be seen to be surrounded by capillaries. The tissue has the appearance of an internally secreting gland and resembles in fact the zona glomerulosa of the adrenal cortex. This resemblance is not an accidental or superficial one. For it is a noteworthy fact that the lipoids disappear also from the adrenal cortex as the result of withholding vitamines from the diet. There is, however, this difference—that the cortex is never completely freed from its lipoid content. A little always remains even when the surrounding peri-adrenal tissue has lost all its lipoids. The significance of this resemblance between the glandular adipose tissue and the adrenal cortex will be discussed presently. At present we wish to emphasise the following points. When, as the result of withholding vitamines from the diet, the animals become emaciated, the ordinary adipose tissue loses its fat deposit and disappears *qua* tissue, the cells reverting to the connective-tissue type of cell. But the glandular adipose tissue persists *qua* tissue and retains its glandular character which now in fact becomes unmistakable. Its morphological appearance, its

highly vascular character, the independence of its lipoid content from the diet as compared with the ordinary fat metabolism and its functional relationship to the thyroid and adrenal glands, which will be discussed in great detail below, make it difficult to avoid the conclusion that this organ is an internally secreting gland. In any case since this tissue has been discovered and forgotten again and again, it seems desirable to emphasise its importance and its essentially glandular character by calling it "the lipoid gland" or "cholesterin gland."

Since this lipoid gland tissue is widely distributed throughout the body, some of it being in close connection with the large blood-vessels, its function cannot be determined by extirpation. But since the death of an animal on a vitamine-free diet coincides with an exhaustion of the contents of the gland, the conclusion seems obvious that the gland is itself rich in at least one of the vitamines and supplies the animal with it when the external supply is cut off. Experiments, which are at present in progress to test this point, appear to confirm this conclusion with reference to the fat-soluble accessory food-substance.

These observations raise a question of general importance in connection with the ætiology of the deficiency diseases. For they suggest that disturbances of the functional activity of this gland may constitute an important factor in eliciting the disease.

ON THE FUNCTIONAL SIGNIFICANCE OF THE GLANDULAR ADIPOSE TISSUE IN RELATION TO THE ADRENAL GLAND.

The adrenal gland lies embedded in the glandular adipose tissue, which is sometimes very firmly adherent to the adrenal gland itself. If a section of adrenal cortex with the surrounding fat is examined in polarised light, the double refracting material outside and inside the adrenal cortex has the same appearance. In fact in many cases a continuous band of double refracting material stretches across the field, and only on turning the analysing prism is it found that this band lies partly inside and partly outside the adrenal. Even if one did not know that this material contained cholesterin compounds both inside and outside the adrenal cortex, one would have no hesitation in concluding from the appearance of the picture in polarised light that the anisotropic material in the adrenal cortex is essentially the same as that in the periadrenal lipoid gland, and that a close functional relationship exists between the two. The adrenal cortex is known to contain a phosphatide, free cholesterin and cholesterin esters in addition to ordinary fat and fatty acids, and the same groups of lipoids may therefore be presumed to be present also in the glandular adipose tissue. The exact chemical nature of the phosphatide present in the adrenal cortex is not known.

The occurrence of cholesterin compounds in normal tissues is curiously

restricted. Hitherto the adrenal cortex has been the only organ in which cholesterin compounds have been found to be present normally and constantly in considerable amounts. The corpus luteum is also stated to contain cholesterin compounds in certain stages of its development. To these two tissues may now be added the glandular adipose tissue. Both the adrenal and the ovary lie embedded in this tissue. While, therefore, the power to form or to serve as a store of cholesterin compounds is restricted to a very small group of cells in the organism, these few groups of cells happen to have a very close anatomical relationship. One cannot escape the suggestion that the glandular adipose tissue and the adrenal cortex are functionally related.

In this connection reference may be made in passing to the pathological conditions in which accumulations of cholesterin compounds occur, namely in the aorta in the experimental atheroma produced by feeding with cholesterin and in the large white kidney. Again, it is difficult to believe that it is a mere accident that both these organs have an intimate anatomical relationship with the glandular adipose tissue which normally stores and perhaps forms these substances.

This conception is not intended to imply that cholesterin fat tissue is equivalent to the adrenal cortex in every respect, but to suggest that they have a common or similar function in reference to the metabolism of cholesterin and the other lipoids present in the cortex, and to emphasise the necessity of taking into consideration the condition of this lipoid gland in these conditions in which changes in the lipoid content of the adrenal gland are observed. This conclusion is confirmed by the facts, already referred to above, that both the lipoid gland and the adrenal cortex retain their load of lipoid under those conditions under which the ordinary adipose tissue disappears, and that they both lose it when vitamines are withheld from the diet.

The recognition of the importance of the glandular type of adipose tissue throws fresh light on the metabolism of cholesterin, and more especially on the changes which occur in different conditions in the lipoids of the adrenal cortex. Although the exact function and destination of the cortical lipoids remain obscure as yet, certain definite conclusions can now be drawn with reference to their origin and to the significance of their changes in the cortex.

The work of Landau (10) (11), McNee (12), Hueck (15), Krylow (14) and others has shown that the lipoids do not represent the internal secretion of the cortex, as was at one time supposed. They are not formed in the cortex, but are taken up by the cortex from the outside. The adrenal cortex is therefore not the central organ regulating the metabolism of these lipoids and of cholesterin in particular, but rather the channel through which these substances pass to their destination, if not actually their destination. It remains to be seen whether the glandular adipose tissue represents the organ regulating the metabolism of these lipoids.

That the direction in which these substances travel in the cortex is from

the periphery of the cortex towards the medulla has already been stated by us in a previous publication (16). The source of these lipoids is very clearly indicated in the condition of avitaminosis described in this paper. The adrenal cortex may be found still carrying its full load of lipoid while the surrounding periadrenal lipoid gland is already being depleted extensively. And when in the terminal stage the lipoid gland has completely lost its load of lipoid the adrenal cortex still retains a small amount.

The tenacity with which the adrenal cortex clings to the last remains of these lipoid substances illustrates another important point, namely that the absence of lipoids from the cortex is not compatible with life. Having examined the adrenals of hundreds of mice and rats, which had been subjected to a great variety of different conditions, I cannot recollect ever having seen a cortex extensively depleted of its lipoids except in animals which had died or were dying.

To avoid misunderstandings it may be as well to state that this does not mean that the lipoids always show an extensive depletion when an animal dies. And further it is necessary to draw a distinction between a mere diminution of the lipoid-load which may be found in a great many conditions, and the almost complete disappearance of these substances from the cortex, which is much less frequent.

In our previous observations on the functional activity of the adrenal gland in different conditions (16, 17), we have found that the disappearance of lipoids from the cortex is always accompanied by a paralysis of the gland. This is particularly striking in the condition of avitaminosis, where a subnormal temperature occurs some days before the terminal stages when the animal is obviously ill. This failure to maintain the normal body temperature is not due to an exhaustion of the medulla, which, although partly depleted, still shows a load of adrenin, but to an actual paralysis of the gland. The gland no longer responds to external cold by an increased secretion of adrenin, nor is it possible to produce a pyrexia when β-tetrahydronaphthylamine is injected. This association of disappearance of cortical lipoid and paralysis of the gland with resultant subnormal temperature has also been observed in some (not all) acute bacterial infections, such as gas gangrene (17).

The view is widely held at present that in all bacterial infections there is an extensive disappearance of the cortical lipoid. This view is based on a statement by Elliott, who was one of the first to examine the behaviour of the cortical lipoid in human post-mortem material in different pathological conditions. He stated that it disappeared with great rapidity in all acute fevers. The extensive observations of Landau and of Weltmann (13) show that this statement is too sweeping and that conditions are not so simple. Observations on human material give very conflicting results, and show that in many acute bacterial infections a normal or almost normal load of lipoid may be found post mortem. Nor is it possible to establish a relation between the

nature of the bacterial infection and the effect on the cortical lipoid. A clearer insight is gained by observations on experimentally induced infections (17). It is then found that the disappearance of the lipoid is only a terminal phenomenon, which is preceded sometimes by a flooding of the cortex with lipoids. It is not the result of the bacterial infection as such, but occurs when the bacterial toxins have exhausted the adrenal gland. Thus a streptococcal infection which produces a circumscribed abscess but does not kill the animal does not produce a disappearance of the cortical lipoids or an exhaustion of the medulla. But when, with the same strain of streptococci, a cellulitis is produced by means of the kataphylactic phenomenon, the animals die, and the adrenals show an exhausted medulla and a cortex almost completely depleted of lipoids.

Whether all bacterial toxins have the same effect on the cortex remains yet to be determined. In a few experiments with tetanus no such effect has been observed. But even assuming that most bacterial toxins show this effect under experimental conditions, it can now be readily understood why human material gives conflicting results. In human post-mortem material the effect will vary with the actual cause of death, whether it has resulted from the pure action of the bacterial toxins, or whether it has been an accidental result such as asphyxia in diphtheria, hæmorrhage, or a toxic action on the heart or nerve centres.

A discussion has arisen as to whether the cortex or the medulla constitutes that part of the adrenal gland which is essential for life, and whether the symptoms following removal—or disease—of the adrenal are due to loss of cortex or to loss of medulla. Our histological observations on the adrenal gland (16) in different conditions of functional activity have shown that activity of the medulla is always accompanied by changes in the cortex, of which the changes in the lipoid are but one aspect, and that the two parts of the gland "act together, and are not, as has been suggested, two organs functionally quite independent of each other." We have only recently become aware that similar conclusions have been arrived at by Landau (11) and by Hueck.(15) Thus the latter writes: "For the normal functioning of the adrenal, medulla and cortex are equally important, and disease of one part acts not only through the diminution of its specific functions, but primarily through disturbance of the synergism."

SUMMARY.

In all types of mammals which have been investigated there exists a glandular type of adipose tissue histogenetically distinct from the ordinary adipose tissue. It has been described under various names, *e.g.* "primitive fat organ," "fat gland," "hibernating gland," "brown fat," "interscapular gland." In all species it has in the embryo a characteristic gland-like structure. In some species (white rat, tame mouse, hibernating animals) it

retains this characteristic histological structure. But in most species the tissue acquires the appearance of ordinary adipose tissue soon after birth, so that the process has been looked upon as one of the stages leading to the formation of the adipose tissue of the adult organism.

It is shown that this tissue, which is very vascular, is functionally distinct from ordinary adipose tissue. The fatty material which it contains is rich in cholesterin compounds and other lipoids in addition to ordinary true fat. This load of lipoid is retained under conditions which bring about the disappearance of the ordinary adipose tissue. Evidence is given to show that it is functionally related to the thyroid and adrenal glands. A particularly close relationship exists between the lipoids of this tissue and those of the adrenal cortex. The significance of the changes of the lipoids in the adrenal cortex is discussed. The inter-dependence of the cortex and medulla of the adrenal gland for the normal functioning is again emphasised.

If vitamines are completely withheld from the diet the lipoids disappear from the adrenal cortex and from this tissue, which persists, however, as a tissue and exhibits the appearance of a very vascular endocrine organ. The existence of this tissue throws fresh light on the problem of deficiency diseases, since disturbance of the functional activity of this gland-like tissue will have to be considered as a factor in the ætiology of these diseases.

In view of its appearance, its function and its importance it is proposed to call this glandular type of adipose tissue the "lipoid gland" or "cholesterin gland."

REFERENCES.

1. Carlier.—*J. Anat. and Physiol.*, 1893, **27**, 508.
2. Cramer, Feiss and Bullock.—XVII Internat. Cong. Med., London, 1913. Sect. of Pathol. Part ii, p. 183.
3. Hammar.—*Arch. f. mikrosk. Anat.*, 1895, **45**, 512.
4. Batty Shaw.—*J. Anat. and Physiol.*, 1902, **36**, 1.
5. Hatai.—*Anat. Anzeiger*, 1902, **21**, 369.
6. Bonnot.—*J. Anat. and Physiol.*, 1908, **43**, 43.
7. Curling.—*Med.-Chirurg. Transact.*, London, 1850, **33**, 303.
8. Hilton Fagge.—*Ibid.*, London, 1871, **54**, 154.
9. Shattock.—*Proc. R. Soc. Med.*, 1909, **2**, Pathol. Sect., p. 252.
10. Landau.—*Deutsche med. Wochenschr.*, 1913, **39**, 546.
11. *Idem.*—17 Verhandl. d. deutsch. pathol. Gesellsch., 1914, p. 144.
12. Landau and McNee.—*Beitr. z. pathol. Anat. u. z. allg. Pathol.*, 1914, **58**, 667.
13. Weltmann.—*Ibid.*, 1913, **56**, 278.
14. Krylow.—*Ibid.*, 1914, **58**, 469.
15. Huecк.—17 Verhandl. d. deutsch. pathol. Gesellsch., 1914, p. 149.
16. Cramer.—Sixth Sci. Rep. Imp. Cancer Res. Fund, London, 1919 (Taylor and Francis), p. 1.
17. Bullock and Cramer.—*Ibid.*, p. 23.

DESCRIPTION OF FIGURES.

FIG. 1.—Scapular lipoid gland of rat. Schridde preparation unstained. × 25 diam. The plate illustrates the lobular arrangement of the tissue as well as the differences in the size of the globules in which the lipoid material may be arranged within the cells. The lobules in the upper part of the plate consist almost entirely of the multiglobular variety; the lower part shows numerous uniglobular cells mixed with multiglobular cells. (Microphotograph.)

FIG. 2.—Human perirenal lipoid gland. From a child. × 240 diam. The left half of the plate is from a Zenker preparation, stained with hæmatoxylin and van Gieson. The right half from a Schridde preparation, unstained, of the same material. (From a drawing.)

FIG. 3.—Thoracic lipoid gland. Rat. Zenker preparation. Hæmatoxylin and van Gieson. × 250 diam. *Ao.* = aorta; *Adv.* = adventitia; *L. G.* = lipoid gland. (From a drawing.)

FIG. 4.—Perirenal lipoid gland. Mouse. Kolster preparation, stained for Altman's granules with aniline acid fuchsin and picric acid. × 1400 diam. The figure shows the relation of the mitochondria in the cells of the tissue to the lipoid globules. The latter have been dissolved out and appear in the figure as empty spaces. (From a drawing.)

FIG. 5.—Scapular lipoid gland. From a rat, dying as the result of being kept twenty-five days on a diet completely free from vitamines. Fixed in osmic vapour. Stained with Heidenhain's iron-alum hæmatoxylin after a previous treatment with 10 per cent. hydrogen peroxide in 80 per cent. alcohol. × 1200 diam. The tissue, which is now entirely free from fats or lipoids, is composed of large cells filled with granular protoplasm (mitochondria) and is very vascular and deeply congested. To the naked eye the tissue appeared purple when the rat was killed. (From a drawing.)

FIGS. 2 to 5 were drawn with a camera lucida in the laboratory by W. Pilgrim.

THE MYCELIAL AND OTHER MICRO-ORGANISMS ASSOCIATED WITH HUMAN ACTINOMYCOSIS.

LEONARD COLEBROOK, M.B., B.S.Lond.,

Assistant, Bacteriological Department, National Institute for Medical Research, Hampstead.

Received for publication July 13th, 1920.

Seventeen cases of actinomycosis came under treatment in the Inoculation Department, St. Mary's Hospital, during the years 1911–1914, and several more have been seen by the author since that time. Some observations made in connection with the earlier cases were being prepared for publication in 1914, but had to be laid aside in favour of military duties. This report has now been brought up to date and divided into two parts, of which one, containing the laboratory findings, is here presented.

Only those cases have been regarded as actinomycosis which showed suppurative lesions, the pus of which contained "granules" visible to the naked eye, composed of a feltwork of filamentous micro-organisms.

In restricting the use of the term to these cases, I have practically adopted the standpoint of Homer Wright(1), who first showed that such a limitation had the practical merit of isolating a group of infections which are attributable —in the great majority of cases, at any rate—to one and the same species of micro-organisms. In this way we avoid much of the confusion which has hitherto surrounded the subject of mycelial infections.

It is recognised that this arbitrary limitation is probably not entirely defensible, inasmuch as it would include certain distinct conditions such as "Madura foot" and other less common mycoses. When knowledge of the mycelial organisms is further advanced a more exact classification may be possible.

During the period covered by my observations of this disease, it has happened several times that mycelial organisms, showing no such aggregation into naked-eye granules, have been found in the discharge from various suppurative lesions, *e.g.* from an old empyema cavity, an acute suppuration in the region of the parotid gland, and an abscess of the breast. These organisms, when cultivated, were found to have characters quite different from those of *Actinomyces bovis* as described below. Cases of this kind have been excluded from the present series.

The presence of "clubs" ("ray-formation") at the periphery of the granules was also included by Homer Wright as a *sine qua non* in making the diagnosis of actinomycosis. In this I have not strictly followed him, for the reason that "clubs" were not looked for in several of the earlier cases of my series. In all the later cases (*i. e.* in at least 75 per cent. of the whole) these structures have been recorded as present. It would seem unlikely, however, that "club"-formation will prove a valuable distinguishing characteristic, since they have been shown, in recent years, to occur in at least two conditions quite distinct from actinomycosis, viz. in actinobacillosis, a purely bacillary infection of cattle (Lignières and Spitz[2]), and after the injection of killed tubercle bacilli into animals (Eastwood[3]).

NATURE OF THE CASES AND SOURCE OF CULTURES.

The 28 cases of actinomycosis which have come under my notice comprise 11 cases of the cervico-facial type, in whom the infection was first manifested on the lateral aspect of the jaw, and afterwards extended upwards or downwards, or in both directions; 7 thoracic cases, in whom the affection apparently originated in the lung-tissue or pleura; 8 abdominal cases, the majority of them having the original lesion in the appendix, or, at any rate, in the region of the cæcum; 1 case in whom both abdomen and thorax were involved (this case was unique in that the disease first manifested itself in two separate lesions, so far apart as the shoulder and the iliac fossa; the focus in the iliac fossa was reported to be extra-peritoneal, and there was no indication of an intra-thoracic origin for that on the shoulder); and 1 case in whom the back of the hand was the seat of the infection.

The course of the disease in nearly all the cases was very slow, extending over several months or even years. Of the 11 cervico-facial cases only 1 died (of cerebral abscess), and 9 are known to have recovered; of the 7 thoracic cases 5 are known to have died of the disease, and 2 have not been traced; of the 8 abdominal cases 6 have died, 1 recovered, and 1 is at present very ill; the case in whom both thorax and abdomen were involved died after three years' illness; the hand case made a rapid recovery.

Some notes on the treatment of this series of cases are being prepared for publication. Several of the cases have already been described by Mr. V. Z. Cope[4], to whom I am indebted for sending them to me, and for help in connection with their treatment.

In addition to the material derived from these cases, I have obtained cultures from the pus of three cases, kindly sent to me by Dr. Mervyn Gordon.

Both for diagnosis by films, and for cultural purposes, it has proved necessary to isolate the actual granules from the pus; separate mycelial filaments were very seldom found in film preparations of pus taken at random, and still more seldom was a growth of the organism obtained from such random samples.

When the granules, usually yellowish-white, about the size of a small pin's head, were not easily detected in the discharges on the surface of the wound, nor in the sample sent to the laboratory for examination, a small quantity of pus was vigorously shaken in a test-tube of water. All the ordinary elements of the pus were emulsified by this means, but the granules remained intact and were readily seen as they sank to the bottom of the tube, from which they were collected in a pipette.

To verify its mycelial structure and detect the "club-" or "ray-" formation, a granule was transferred to a microscopic slide, lightly crushed under a cover-glass, and examined, unstained, with a $\frac{1}{6}$th or $\frac{1}{8}$th objective. For more detailed examination another granule was crushed more thoroughly between two slides, stained by Gram's method and counterstained with weak carbol fuchsin.

To obtain cultures of the mycelium several granules were obtained as above, thoroughly washed in one or more changes of sterile water or broth in order to free the mycelium as far as possible from contaminating bacteria, and then crushed in a drop of fluid between sterile microscopic slides. Two glucose agar plates (P_H about 7·4) were inoculated from the mycelial suspension thus prepared, one being incubated aërobically, the other in a jar from which the oxygen was removed by the palladium chloride method (McIntosh and Fildes ([5])). Before this anaërobic method was introduced, anaërobiosis was obtained usually by exhaustion of air, or by exhaustion plus absorption of oxygen with pyrogallic acid and soda. The results, however, when these latter methods were employed, were unsatisfactory in that growth often failed to occur, especially primary growths. I therefore relied more upon suspension cultures. These were made either (*a*) by aspirating the melted agar, after inoculation with an emulsion of a crushed granule, into a long piece of glass tubing, previously drawn out into a capillary at each end to permit of sealing with the inclusion of a negligible quantity of air (method of Vignal), or (*b*) more simply, by pouring the inoculated agar into a narrow test-tube and covering the top of the column with a layer of liquid paraffin or vaseline.

The mycelial colonies were easily recognised in such suspension cultures, and could be isolated by cutting across the glass tube and agar column.

The method recently described by Dr. Mervyn Gordon([6]) of planting a granule into a tube of blood-broth under oil promises to secure primary cultures with greater ease than either of the above procedures. It proved successful in my hands with three of four samples of pus recently sent to me by Dr. Gordon, and in one instance a growth was obtained by this means when the surface culture failed to grow the mycelium. The method has one disadvantage, viz. that if pure cultures of the mycelium are required, these may be somewhat difficult to obtain from a primary mixed culture, in which the contaminating organisms frequently present (*e. g.* staphylococci, streptococci, and coliform bacilli) grow so much more quickly and profusely than

the mycelium. For this subsequent isolation of the mycelium anaërobic plate-cultures are to be recommended, *the mass of mycelial growth being first washed in several changes of sterile water* in order to free it, as far as possible, from contaminating organisms.

THE MYCELIAL ORGANISMS.

The mycelial organisms isolated will first be dealt with, and afterwards certain bacteria found in association with these.

Growths of these organisms have been obtained from the granules of 27 cases and have ultimately been freed from associated bacteria. Three strains must be disregarded owing to insufficient records of their characters having been kept. Of the 24 remaining, 21 strains showed a general resemblance to each other, and may therefore be considered as one group (A). Two strains resembled the members of this group in respect of their morphology and preference for anaërobiosis, but differed somewhat in the character of their growths (Group B). One strain was frankly different from the common type (Group C). So far as memory serves to recall, the three strains which have been left out of account all conformed to the common type.

Group A.

The 21 strains of this group were filamentous organisms, the filaments being slender (0·5 to 1·0 μ), showing true branching, and seldom running straight for more than 20 to 30 microns. They retained Gram's stain, but often somewhat irregularly, so that deeply stained portions of the threads alternated with lightly stained portions (*vide* Fig. 1); at other times the deep staining was confined to mere dots within the thread. Recently Salomonsen's wet staining method has been applied to several strains with very satisfactory results, using fuchsin with carbolic acid and glycerine. A striking feature brought out in these preparations was the presence of very deeply stained dots at irregular intervals, sometimes 4 or 5 μ apart, at other points quite close together. Terminal thickenings and others of spindle shape have sometimes been seen along the threads, but neither spores nor the septate spore-bearing hyphæ recently described by Dreschler(7), in connection with the actinomyces of soil, have so far been demonstrated. No acid-fast strain has been met with. Growth of the organisms was always slow, being manifested only after three to eight days in primary cultures and two to six days in subcultures. It was never obtained at 22° C.

A strong preference for anaërobic growth was shown by all strains, but several were found to have become capable of some degree of aërobic growth after a period of frequent subculturing in broth under a column of liquid paraffin. Occasionally even primary aërobic cultures yielded one or two colonies after ten to fourteen days. Such aërobic surface growths always tended to grow down into the medium. With suspension cultures in glucose

FIG. 1.—Crushed actinomycotic granule, showing mycelium of actinomyces surrounded by closely packed masses of *B. actinomycetum comitans.*

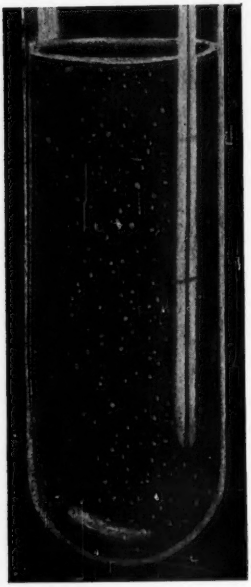


FIG. 3.—Broth culture of *B. actinomycetum comitans,* showing pin-point colonies adherent to the glass.

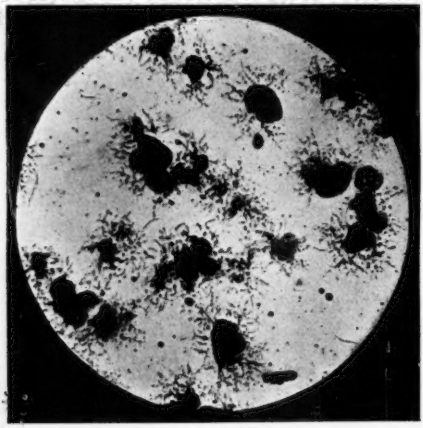


FIG. 2.—Surface colonies of *Actinomyces bovis* on glucose agar, 6 days old. × 55.

agar, it was noticed on several occasions that a dense band of small colonies would develop about half an inch below the top of the column, while in the deeper parts of the column the growth was much more scattered.

Cultures of all strains were obtained on ordinary nutrient agar or broth, but were much more luxuriant if ½ or 1 per cent. of glucose had been added to the medium.

A series of tests with nutrient broths of different hydrogen-ion concentrations showed that when the P_H lay within the range 6·7 to 7·7 good growths were obtained, and that the optimum was from 7·3 to 7·6.

Surface cultures were usually obtained without difficulty when the palladium chloride method of securing anaërobiosis was employed, otherwise they were frequently unsuccessful. Young colonies, under low magnification, showed a margin of irregularly radiating processes (Fig. 2). When more fully grown they formed dull white, heaped-up masses, which were very adherent to the medium and were not easily broken up. The typical appearance of a fully-grown surface culture is well shown in a photograph published by Wolff and Israel[8] (Plate I, fig. 1).

In suspension cultures the colonies were opaque and quite irregular in shape, suggesting rather the appearance of a cauliflower in miniature. They were thus in sharp contrast to the regularly formed lenticular colonies of staphylococcus, with which they were often mixed in primary cultures.

In fluid media (usually glucose broth) growth occurred always at the bottom of the tube in the form of opaque, dull white masses, rather suggestive of bread-crumbs, which were not easily broken up. The broth remained clear, but would sometimes show a flocculent deposit when its reaction became acid after five to ten days.

In tubes of uncooked white of egg, or a mixture of yolk and white, little if any growth was obtained.

In human serum, of which the anti-tryptic power had been neutralised by the addition of trypsin, a fairly free growth occurred. Very little growth was obtained in unaltered fresh serum.

The viability of these strains was somewhat slight. They were always killed by exposure to 60° C. for an hour, and surface cultures appeared to be sensitive also to desiccation. Even when frequently subcultured they were apt to die out.

Cultures on egg medium, when kept moist, have remained alive for four to six weeks, and one strain lived for four weeks on the surface of glucose agar when kept in the ice-chest.

Glucose broth cultures did not usually survive longer than fourteen days at 37° C.

Identity of the strains of Group A with the Actinomyces bovis *of Wolff and Israel.*—Comparison of the characters described above with those attributed by Wolff and Israel[8] to the organisms which they isolated from two cases of

actinomycosis, and also with the account given by Homer Wright of the 13 strains isolated by him in America, leaves little doubt that the 21 strains here in question showed a very close resemblance to the strains of these authors. Without entering upon vexed questions of nomenclature, I have therefore followed the lead of Wolff and Israel and applied to these 21 strains the name "*Actinomyces bovis*." This seems to have been introduced in 1877 by Harz, who first described an organism apparently identical with that of Wolff and Israel.

These authors noticed a tendency of their strains to revert to a chiefly bacillary habit of growth in artificial media. One or two of my strains have shown this tendency, but it has not been the rule. Wolff and Israel also seem to have found their strains somewhat more hardy than mine have proved; this, however, may be accounted for by the fact that these authors largely used egg medium, whereas I have employed chiefly media containing a considerable amount of sugar.

Group B.

Unfortunately my notes of these two strains, which were isolated just before the war, are not adequate for a satisfactory description. Both were recovered from clinically typical cases of actinomycosis, with granules in the pus. In one case the illness started as an acute attack of appendicitis.

Both strains resembled *Actinomyces bovis* in their preference for anaërobiosis, their slight viability, and in their morphological characters when first isolated. One of them differed chiefly from the strains of Group A in the character of its growth on the surface of glucose agar. The second strain grew badly on glucose agar, and in glucose broth produced a uniform turbidity with a sediment—not bread-crumb-like masses. On blood agar the mycelium grew fairly readily in small greyish colonies, which were slightly raised and did not stick to the medium. No aërobic culture was obtained.

The second and third generation in broth showed almost entirely bacillary forms.

Group C.

This strain (Taylor) was recovered from a granule found in pus from an old-standing thoracic sinus. The illness had commenced with empyema four years previously, and this had been followed by several abscesses in the chest-wall. When seen by the author the patient was discharging occasional granules from a lesion near the xiphisternum. His general health was very good.

A film preparation of a granule showed a mass of mycelium of which the elements ran rather straighter than those of *Actinomyces bovis*, but were, in other respects, similar to that organism. Unfortunately the granule was not examined for "ray"-formation.

Cultures yielded a single colony of an *aërobic* mycelial organism, this colony developing in the water of condensation. A few colonies of diphtheroid bacilli

also grew. Subcultures of the mycelial colony on glucose agar gave several large flat rosettes (3 to 5 mm. in diameter), which grew deeply into the medium and were extremely tough. In gelatine at 37° C. they formed a large, spongy mass. The cultures died out during the writer's absence for three weeks, and so were not examined further.

THE GEOGRAPHICAL DISTRIBUTION OF *ACTINOMYCES BOVIS* INFECTIONS.

The predominance of *Actinomyces bovis* in the author's series of cases is of interest as bringing the experience of English cases into line with that of several other countries. As stated above, Homer Wright cultivated this organism from 13 consecutive cases of human actinomycosis in America. Of 29 Japanese cases from which cultures were obtained, 28 gave strains of this type (Shiota [9]); Schukewitsch [10] isolated 22 similar strains derived from 25 cases in Russia; and Harbitz [11] obtained 20 strains in Norway. Finally, Pinoy [12], working in Paris, described 13 strains out of 16 isolated from cattle, and 2 out of 3 from human actinomycosis, as agreeing with this type, to which he gives the somewhat cumbrous name "*Cohni-streptothrix Israeli.*"

THE SOURCE OF THE INFECTION IN HUMAN ACTINOMYCOSIS.

The established idea that the "ray fungus" is conveyed to man from vegetable sources, *e.g.* grasses, soil, etc., should be viewed with considerable scepticism.

This concept appears to have been based upon the view that the common cause of actinomycosis is an *aërobic* organism of the type described by Bostroem [13] and others, which is more or less akin to certain moulds recoverable from vegetable sources. The work of Homer Wright and others quoted above has shown, however, that the infective agent in most cases of actinomycosis is not of this type, but is the *anaërobic* organism here referred to as *Actinomyces bovis* (Group A of the author's series); and no one, so far as I am aware, has yet recovered an organism of this latter type from any vegetable or other "natural" source, *i. e.* outside the animal body. When we consider the slight viability of *Actinomyces bovis*, its apparent inability to form spores or to propagate the mycelial generations except at body temperature, there seems little reason to expect that it will be recovered from "natural" sources.

With a view to testing this hypothetical infection by the "fungus" when conveyed into the tissues on vegetable material, a few experimental inoculations of rabbits were carried out. Sterile oats were impregnated with a fluid culture of *Actinomyces bovis*, and were then introduced into the pleural and peritoneal cavities. Actinomycotic infection was not established in any instance.

Finally, before leaving this view of the question, it may be remarked that only 1 of the 28 cases of which I have had experience gave a history that at

all suggested this traditional mode of infection. The patient was a boy under the care of Mr. Cope, who has reported it in his paper (Case 1). A piece of grass was discharged in this case from an abscess at the angle of the jaw.

Another, and seemingly more credible, view as to the origin of the disease has recently been gaining ground. It does not attempt to explain from what source or in what way *Actinomyces bovis* is originally conveyed to the human body, but suggests that it is a frequent inhabitant of the alimentary tract of man, and that in certain occasional circumstances, which are not yet clear, it is carried into the tissues and is enabled to establish itself there.

The evidence in favour of this view is as follows:

Lord ([14]) has called attention to the fact that in carious teeth, and also in tonsillar crypts of normal persons, one may frequently find filamentous organisms having the morphology and staining reactions of *Actinomyces bovis*; and further, he has succeeded, by inoculating such material intraperitoneally into animals, in reproducing granulomatous lesions which resembled those of actinomycosis in that they contained granules composed of a feltwork of the mycelium and surrounded by typical clubs.

The present writer also found Gram-positive filaments not unlike those of actinomyces in each of six carious teeth examined, but was not able to carry the investigation further at that time. It would seem very desirable to isolate these filamentous organisms from a series of teeth and determine whether they can be identified with *Actinomyces bovis* by serological tests.

In support of the possible dental origin of the disease in certain patients, one of the cases reported by Mr. Cope and included in the present author's series was of peculiar interest. The patient had injured his knuckle by knocking out an opponent's tooth three months before coming to hospital. An acute infection ensued, with lymphangitis; this subsided, and was followed, after a few days, by the development of a subacute actinomycotic lesion.

A very similar case has recently been reported in America (McWilliams ([15])).

AGGLUTINABILITY AND AGGLUTINOGENIC PROPERTIES OF *ACTINOMYCES BOVIS*.

The author's observations in respect of these properties of actinomyces are very incomplete, but inasmuch as very little has been written on the subject hitherto, it is thought perhaps worth while to record the following points in summary form: (*a*) The serum of heavily-infected patients usually gives a coarse agglutination with suspensions of *Actinomyces bovis*. Such sera were often effective in a dilution of 1 in 500, sometimes in dilutions as high as 1 in 4000.

Most of the patients with relatively slight infections, *e.g.* the cervico-facial cases, failed to show such agglutination. The reaction will probably be of little use for diagnosis, since cases usually have open lesions in which the mycelium can be found by the time the serum has developed agglutinating power.

(*b*) Similar coarse agglutination is obtained with the sera of rabbits inoculated with a vaccine of *Actinomyces bovis*. Two doses of 500 and 1000 million mycelial fragments under the skin, followed by three doses of 500, 1000 and 2000 million fragments respectively into the blood-stream, at two or three days' interval, sufficed to obtain a serum agglutinating up to a dilution of 1 in 1000.

(*c*) Fine agglutination, occurring very slowly, is sometimes seen with normal sera, even when diluted 50- or 100-fold, but there is usually no difficulty in distinguishing between this and the coarse agglutination which occurs within one hour or two hours at 50° C. with the sera of infected persons or animals. Spontaneous agglutination of the suspension of actinomyces was also noticed at times, particularly if a 0·85 per cent. or 1 per cent. salt solution were employed. Suspensions in weaker salt solutions have been free from this defect.

(*d*) The most satisfactory suspensions of actinomyces for agglutination tests were prepared by thoroughly washing the growth obtained from a broth culture, drying it on filter-paper, and then rubbing up the flaky powder in a mortar with 0·4 per cent. sodium chloride solution.

Serological types of Actinomyces bovis.—The preparation of agglutinating sera to several strains of this organism, with a view to testing them against all available strains, had just been begun when the work was interrupted by the war. It has not yet been determined, therefore, whether there exist several different serological types of *Actinomyces bovis*.

Two such sera have been kept, and others are in course of preparation.

The sera of infected patients were tested from time to time against several strains, and showed a certain amount of cross-agglutination. The results were, however, sometimes suggestive of serological differences. One such observation is perhaps worth recording: The serum of a patient, D. R—, although it agglutinated the homologous strain, failed to agglutinate another strain, A. W—; while the serum of the patient A. W— agglutinated the strain D. R— as well as the homologous one. This serum also agglutinated four other strains.

ON CERTAIN BACTERIA COMMONLY FOUND IN CLOSE ASSOCIATION WITH ACTINOMYCES.

Actinomycotic granules are usually surrounded by an envelope of closely packed leucocytes. If this envelope is washed away by shaking the granules in water, and the core remaining is then crushed between two slides, stained by Gram's method and counter-stained with weak carbol-fuchsin, the felt-work of Gram-positive mycelium will often be seen upon a background which has taken the counter-stain, but in which no detailed structure can be made out; it appears as a pink or red blur.

This counter-stained background is in reality a densely compacted sheet

of very minute Gram-negative cocco-bacilli, as may be ascertained by following it out to a point where the sheet has been lacerated in crushing the granule. At such a point it will frequently be possible to trace the fusion of the multitude of individual cocco-bacilli (*vide* Fig. 1).

My attention was first directed to these organisms in 1912 in finding that a multitude of tiny colonies, quite distinct from the mycelial colonies, had grown in an agar suspension inoculated from a crushed granule which had shown only mycelial elements on casual examination of a film. A smear of these tiny colonies showed them to be composed of Gram-negative cocco-bacilli. On going back to the film made from a granule and looking carefully at the thinner parts of it, great numbers of lightly stained Gram-negative elements could be made out and their fusion into dense sheets could be determined at some points.

Since that observation was made the presence or absence of these organisms in the granules has usually been noted. Out of thirty patients whose granules have been thus examined, they have been found in no less than twenty-four, and in more than ten instances the organisms have been cultivated as well as seen in films. Usually, but not invariably, they were present when the patient first came under treatment, and persisted throughout the course of the disease; in one case they were not found until the patient had been under observation for nearly a year, but from that time were constantly present. As a rule they occurred, as described above, in the form of dense masses, giving the appearance of a matrix about the mycelial feltwork of the granules; I have never detected them free in the pus.

Morphology.—When seen in films of crushed actinomycotic granules, and also in smears made from cultures, these minute organisms most commonly have the form of micrococci (diameter about 0·3 to 0·5 μ), or very short round-ended bacilli (0·8 to 1·5 μ in length). On this account they were at one time referred to in the laboratory as "*Micrococci pseudo-melitensis*."

Longer bacillary forms are also frequently seen and may perhaps represent a distinct type, but no definite evidence of this has been obtained.

The formation of short chains has occasionally occurred in broth cultures, but seems to be exceptional. Capsules have not been demonstrated. The organism is non-motile.

Gram's stain is never retained, and if neutral red is employed as the counter-stain the organism may not be detected, for it takes that dye very badly. Carbol fuchsin is a better counter-stain, but even that is not taken up so readily as by most bacteria. With Leishman's modification of Romanowsky's stain a tendency to bi-polar colouring has sometimes been noted.

Cultural characters.—The outstanding characteristic of this organism is the tenacity with which the individuals cling to each other. This has already been seen in connection with cultures of the microbe in the body, *i. e.* in the actinomycotic granule, and it is no less manifest in cultures grown outside the

body. The colonies produced on the surface of agar are not only very difficult to break up, but also to remove from the medium. And in fluid cultures growth is characterised by the formation of star-like colonies which adhere with extraordinary tenacity to the walls of the tube (Fig. 3), so that they are scarcely to be removed by mere shaking.

Growth occurs equally well under aërobic or anaërobic conditions, but is somewhat precarious. Primary cultures from crushed granules have very frequently failed to grow the microbe, possibly because it had been killed by the great concentration of leucocytes around the granule. The successful cultivations, when obtained, were made upon ordinary media, simple nutrient agar or glucose agar; one strain, however, required blood agar at first, and another was certainly favoured by the inclusion of serum in the medium.

No growth has occurred at 22° C.

Surface colonies become visible after twenty-four hours' incubation and usually increase in size for two or three days—seldom, however, exceeding 1 mm. in diameter. They are discrete, hemispherical, and glisten with the lightest suggestion of a blue tinge. As described above, they are dislodged with difficulty from the medium and are not easily broken up.

In broth cultures the colonies develop, as was explained above, on the sides of the tube, not exclusively at the bottom. They are usually about 0·5 mm. in diameter when full grown, but may be slightly larger. After several sub-cultures one strain was observed to give a uniform turbidity in broth.

Glucose, lactose, mannitol, dulcitol, saccharose and dextrin respectively, were fermented by one strain, the only one tested—acid being formed but no gas.

Viability.—This is apparently slight. Cultures are killed by exposure to 52° C. for half an hour, or for two days in the ice-chest. Surface cultures on agar have survived on the bench for two to three weeks, suspension cultures a somewhat shorter time.

Pathogenicity for man.—It is difficult to determine what part, if any, is played by this microbe, as distinct from actinomyces, when both are present in an actinomycotic lesion. The cocco-bacillus has not been found in any lesion independently of actinomyces.

When a living suspension of the organism, which had been frequently subcultured (and was therefore, perhaps, avirulent) was on one occasion introduced into the skin of a normal individual no effect was produced.

The investigation of serum reactions (opsonic and agglutination) in infected patients has not been of much value. Great difficulty is experienced in obtaining satisfactory suspensions for these purposes from the scanty and tenacious cultures. High opsonic values (1·4 to 1·8 approximately) have been recorded on several occasions.

Vaccines of the organism have sometimes induced mild febrile reactions in doses of over 40 millions.

Pathogenicity for laboratory animals.—Of three rabbits inoculated intravenously with a culture of this organism, the quantities varying from 300 to 15,000 millions, two died within twenty hours. The lungs, kidneys and spleens showed some congestion, and the leucocytes in the blood-stream were crowded with phagocyted organisms.

Two rabbits inoculated into the peritoneal cavity and subcutaneous tissue respectively, were killed, and no evidence found of either a local or general infection.

Of six guinea-pigs inoculated intraperitoneally, two died with diffuse peritonitis and some white nodes in the omentum, but no granule-formation was detected. Another died after thirty days with congestion of the tunica vaginalis but no peritonitis. Guinea-pigs inoculated subcutaneously developed an indurated inflammatory reaction which did not go on to abscess-formation.

References to this cocco-bacillus in the literature.—The paper by Wolff and Israel ([8]) (1891), which first described the cultural characters of *Actinomyces bovis*, contains some discussion as to the nature of certain pleomorphic bodies, "possibly micrococci," which had been observed by them as well as by other workers, mixed with the mycelium of actinomycotic granules. It seems unlikely, however, that these "micrococci" can have been identical with the bacteria described above, since the latter are always frankly Gram-negative, whereas Wolff and Israel's "micrococci" are described as Gram-positive. These "micrococci" do not appear to have been cultivated.

The only paper I have met with which describes the same organism as the one here in question is that of Klinger ([16]), who, working in Zurich, found it in the granules of four cases of actinomycosis, always along with actinomyces. He described its cultural characters and proposed for it the name of *B. actinomycetum comitans*, by which it will be referred to hereafter in this paper.

DISCUSSION AS TO THE IDENTITY OF *B. ACTINOMYCETUM COMITANS* AND AS TO THE SIGNIFICANCE OF ITS ASSOCIATION WITH ACTINOMYCES.

The bacterium has not, so far as I am aware, been identified with any other species. Nor has it been met with in any other habitat than the actinomycotic granule.

I suspected at first, on account of certain resemblances in its morphological and cultural characters, that it might prove to be identical with the actinobacillus of Lignières and Spitz. These authors described in 1902 a disease occurring among Argentine cattle which resembled actinomycosis in giving rise to suppurative lesions, the pus of which showed granules with well-marked "ray-formation." The disease, however, differed sharply from actinomycosis clinically by its predilection for attacking lymphatic glands, and, bacteriologically, by the absence of mycelium from the granules, which were composed exclusively of minute bacilli. The term "actino-bacillosis" was applied to the disease, and "actino-bacillus" to the causal organism. It has been shown by

F. Griffith [18] (1914) that the great majority of so-called actinomycosis lesions of cattle in this country are, in reality, due to actino-bacillosis, the bacillus recoverable from them showing a close resemblance to that of Lignières and Spitz. One human case of infection by this bacillus has been reported by Ravaut and Pinoy [17] in Paris. The suspected identity of *B. actinomycetum comitans* with this actino-bacillus has not, however, been established. On examining cultures of the actino-bacillus, kindly shown to me by Dr. F. Griffith, it was found that this microbe produces a much more abundant surface growth on agar, and in broth a uniform turbidity with a scum on the surface.

To the question, What is the significance of the frequent association of the bacterium with actinomyces in human actinomycosis? no answer can at present be given.

The following four hypotheses have suggested themselves and may be briefly discussed: *Hypothesis* 1.—B. actinomycetum comitans *is very widely distributed in nature, and therefore finds easy access as a secondary infection to the open lesions of actinomycosis.* This suggestion may be put aside for the following reasons: The bacterium is a quite unfamiliar species, and has not been met with, so far as I am aware, as a secondary invader of any other open wounds. Further, the microbe has been found, in at least one instance, in the pus of a primary actinomycotic abscess (unopened).

Hypothesis 2.—B. actinomycetum comitans *is widely distributed in nature, but establishes itself only in tissues which have been invaded by actinomyces, the previous growth of that fungus having created conditions peculiarly favourable to the bacterium.* In the expectation that a favourable influence of this kind, if strongly exerted by the growth of actinomyces in the body, might also be evidenced in some measure by its cultivation *in vitro*, the following experiment was performed:

A fully-grown suspension culture of *Actinomyces bovis* in glucose agar was melted down, its alkalinity restored to correspond with that of a control tube, and thereafter sloped. Upon this tube, and a control, *B. actinomycetum comitans* was now planted from a common source. A similar experiment was conducted with liquid medium. In both cases the growth obtained with the medium which had previously yielded a crop of actinomyces did not exceed, but rather fell short of that obtained with the fresh (control) medium.

We have, then, no evidence in favour of this hypothesis, which appears unlikely to offer the solution of the problem.

Hypothesis 3.—B. actinomycetum comitans *occurs along with actinomyces in "nature,"*—i.e. *before the latter gains access to the human body—and the infection is consequently a double one.* The association of the two organisms in nature is regarded, on this hypothesis, as independent of any genetic connection between them. This hypothesis is scarcely yet ripe for discussion, since we do not yet know what is the source of actinomycosis in nature, nor how its transference to man comes about.

Hypothesis 4.—*The association of the two organisms in the human lesions is conceived to depend upon some genetic connection between them.* Such a suggestion is not entirely new. Dunbar [19] has described microscopical and cultural observations which suggest the formation of bacillary organisms from certain algæ. He held the view that cholera-like vibrios, plague bacilli and other organisms actually developed from moulds and algæ.

The question whether a similar concept can be applied to the case of actinomycosis must necessarily be held over for future discussion. The research which such a discussion must draw upon is beset by the especial difficulties which attend all experiments pointing to the transformation of microbic species. And particularly in the case now at issue, where both the bacillus and its colony are so small as to easily escape recognition, most scrupulous care will be necessary in order to disarm the possible criticism that the bacillus has been present in, and carried over from, the cultures of actinomyces employed.

For the present only prefatory observations may be recorded:

(1) The bacilli have been found present in abundance in the granules of an unopened primary abscess. Only this one opportunity has yet occurred to examine material from a primary abscess.

(2) 80 per cent. of cases specifically examined for it have shown the bacilli to be present, but in some of these it has only been found in the discharges at irregular intervals. One case constantly failed to show the bacilli over a period of twelve months.

(3) By direct examination of young growing plate cultures of *Actinomyces bovis* under high magnification, Captain Douglas has occasionally observed the inclusion within the mycelial filaments of small bodies comparable in appearance to the *B. actinomycetum comitans*, and at once suggestive of analogous findings described by Dunbar.

(4) By an indirect method, the investigation of specific antibodies, evidence of the kinship of the two organisms has also been sought, but has not been found. The opsonic power of the serum of an actinomycosis patient was tested to both the ray fungus and *B. actinomycetum comitans*. The serum was then incubated with a thick emulsion of the fungus, which was thereafter removed by centrifugalisation. Upon re-testing the serum with the phagocytic emulsions of the organisms, it appeared that while the opsonic power for actinomyces had been almost entirely lost, that for *B. actinomycetum comitans* remained unchanged.

THE SECONDARY INFECTIONS OF ACTINOMYCOSIS (APART FROM INFECTION BY *B. ACTINOMYCETUM COMITANS*).

An open actinomycotic lesion is very rarely infected only by actinomyces. In the cervico-facial cases staphylococcus will usually be found and sometimes also streptococcus, but these infections have not appeared to play an important

part in the progress of the disease. In the abdominal cases, on the other hand, secondary infection from the intestinal tract frequently occurs, giving to the discharges the extremely offensive smell characteristic of proteolytic digestion by bacteria. Anaërobic bacteria, diphtheroid and coliform bacilli are frequently found in this condition, and since their presence generally implies deep suppurative foci which it is impracticable to drain effectively, a grave degree of toxæmia is apt to occur and a fatal issue is the rule.

SUMMARY.

(1) Cultures of mycelial organisms were obtained from the granules of twenty-four cases of human actinomycosis. Twenty-one of the twenty-four strains conformed in their general characters to the anaërobic type described by Wolff and Israel (*Actinomyces bovis*), and later, in greater detail, by Homer Wright; two strains were also anaërobic organisms but with slightly different cultural characters; and one was a quite different, aërobic type.

(2) All strains of *Actinomyces bovis* which were tested showed coarse agglutination with the serum of heavily infected patients. The serum of rabbits inoculated with the organism also gave a strong agglutination reaction.

(3) The current hypothesis that actinomycosis is directly conveyed to man from vegetable sources or from soil is held to be probably false. Certain evidence is put forward which suggests that the organism is a common inhabitant of the human alimentary tract.

(4) Attention is called to the fact that, in addition to the mycelial feltwork always found in actinomycotic granules, there are frequently immense numbers of minute Gram-negative cocco-bacilli. In 80 per cent. of the cases these organisms have been found at some period of the disease; and in about a dozen instances they have been cultivated on artificial media. They are of unfamiliar type, and have not, so far as is known, been met with in any other circumstances. It was proposed by Klinger, who reported the cultivation of similar organisms from four cases of actinomycosis in 1912, to apply to them the name *B. actinomycetum comitans*. This name is here employed.

The association of *B. actinomycetum comitans* with actinomyces in the granules is discussed but no satisfactory explanation has been arrived at. The findings are somewhat suggestive of a genetic relationship between the bacillary organism and actinomyces, analogous to that which Dunbar claimed to have demonstrated in the case of cholera-like vibrios and algæ.

In conclusion I wish to acknowledge the valuable help given to me by Sir Almroth Wright and Capt. Douglas, under whose guidance many of the cases were treated and the laboratory work carried out.

REFERENCES.

1. Wright, J. H.—"The Biology of the Micro-organism of Actinomycosis," *J. Med. Res.*, 1905, **13**, 349.
2. Lignières, J., and Spitz, J.—"L'actinobacillose," *Réc. de méd. vét.*, 1902. Ref. in *Jahresb. ü. d. Fortschr. i. d. Lehre v. d. pathog. Mikroorg.* (*Baumgarten*), 1902, **18**, 612.
3. Eastwood, A.—'Comparative Histology and Bacteriological Investigations,' R. Commission on Tuberculosis. Final Rep. Part II. Appendix, Vol. 5. London, 1911 (H.M. Stationery Office).
4. Cope, V. Z.—"A Clinical Study of Actinomycosis, with Illustrative Cases," *Brit. J. Surg.*, 1915–16, **3**, 55.
5. McIntosh, J., and Fildes, P.—"A New Apparatus for the Isolation and Cultivation of Anaërobic Micro-organisms," *Lancet*, 1916, **1**, 768.
6. Gordon, M. H.—"A Simple Method of Cultivating the Micro-organism of Actinomycosis," *Brit. Med. J.*, 1920, **1**, 435.
7. Dreschler.—*Bot. Gaz.*, 1919, **67**, 147.
8. Wolff, M., and Israel, J.—"Ueber Reincultur des Actinomyces und seine Uebertragbarkeit auf Thiere," *Arch. f. pathol. Anat. u. Physiol.* (*Virchow*), 1891, **126**, 11.
9. Shiota, H.—"Beitrag zur Kenntnis der menschlichen Aktinomykose," *Deutsche Zeitschr. f. Chirurg.*, 1909, **101**, 291.
10. Schukewitsch, J. J.—"Zur Lehre über die Actinomycose des Rindes," ref. in *Jahresb. ü. d. Leistungen u. Fortschr. i. d. ges. Med.* (*Waldeyer and Pösner*), 1903, **37**, 711.
11. Harbitz, F., and Gröndahl, N. B.—"Die Strahlenpilzkrankheit (Aktinomykose) in Norwegen," *Beitr. z. pathol. Anat. u. z. allg. Pathol.*, 1911, **50**, 193.
12. Pinoy, E.—"Actinomycoses et mycétomes," *Bull. de l'Inst. Pasteur*, 1913, **11**, 929 and 977.
13. Bostroem.—"Untersuchungen über die Aktinomykose des Menschen," *Beitr. z. pathol. Anat. u. z. allg. Pathol.*, 1891, **9**, 1.
14. Lord, F. T.—"The Ætiology of Actinomycosis," *J. Amer. Med. Assoc.*, 1910, **55**, 1261.
15. McWilliams, C.—"Actinomycosis of Phalanx of Finger," *Ann. of Surg.*, 1917, **66**, 117.
16. Klinger, R.—"Untersuchungen über menschliche Aktinomykose," *Centralbl. f. Bakteriol.* (Orig.), 1912, **62**, 191.
17. Ravaut, P., and Pinoy.—"Actinobacillose à forme méningée observée à Paris chêz un Argentin," *Presse méd.*, 1911, **19**, 49.
18. Griffith, F.—"On the Pathology of Bovine Actinomycosis," *J. Hyg.*, 1916, **15**, 195.
19. Dunbar.—'Zur Frage der Stellung der Bakterien, Hefen und Schimmelpilze im System,' Munich and Berlin, 1907 (R. Oldenbourgh).

VARIATION IN AGGLUTINABILITY OF BACTERIA ASSOCIATED WITH VARIATION OF CULTURAL CHARACTERS.

T. J. MACKIE.

From the Bacteriological Department, University of Cape Town.

Forwarded for publication May 3rd, 1920.

MUTATION of organisms belonging to the group of Gram-negative intestinal bacilli has been studied by various observers, but mainly with regard to variation of biochemical reactions. These variations have been observed occurring spontaneously and also under the influence of inhibitory agents in culture medium.

Massini (1) originally described a non-lactose-fermenting Gram-negative bacillus (*B. coli mutabilis*) which on Endo-agar developed lactose-fermenting mutants, represented by red papillæ on the pale colonies of the original strain, and, after further subculture, as red colonies. Penfold (2, 3) also described mutation of *B. coli* types growing on monochloracetic-acid-agar, the variant being characterised by absence of gas production in certain fermentation tests. Such variants, however, show no difference from the original strains as regards their serological characters.

Recently Fletcher (4) has described atypical forms of *B. paratyphosus B* and *B. aertrycke* occurring under natural conditions, which in artificial culture exhibited variation both in cultural and serological characters and reverted to the typical form. The original strains were capsulated bacilli, produced slimy or "mucoid" colonies, and were much less agglutinable than the classical strains; after subculture, however, typical bacilli developed from the mucoid strain, and these reacted to full titre with a specific agglutinating serum. Fletcher has also described the same variation in the case of strains of dysentry bacilli.

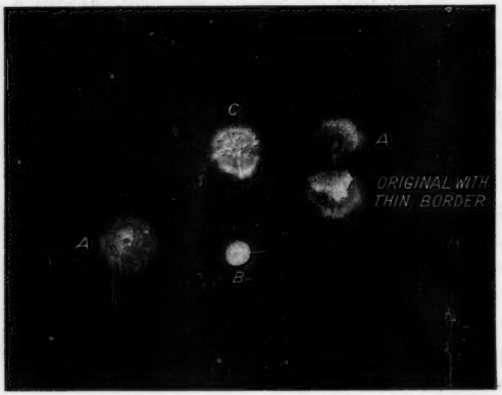
In the course of certain extended observations on the *B. coli* group a similar variation has been noted. It was found that a *B. vesiculosus* (MacConkey) strain which produced on agar plates large, raised, thick, opaque, slimy or "viscous" colonies, and grew in the form of a thick, white, glistening, viscous growth on agar-slope cultures, when grown on agar containing brilliant green

in such amounts as to produce slight inhibition of growth, developed variants from the original type as regards colony characters; it was also noted that these variations were associated with differences in agglutinability by a serum specific to the original strain.

The general characters of the original strain were as follows: Gram-negative, non-motile bacillus; not liquefying gelatine; fermenting, with gas production, glucose and lactose, but not dulcite, saccharose, adonite, inulin, or inosite; producing indol from peptone; not yielding a Voges and Proskauer reaction; *i.e.* corresponding to *B. vesiculosus* (MacConkey). The colony characters have already been referred to. When plated out on nutrient agar containing 0·06 c.c. of a 1 : 10,000 watery solution of brilliant green to 5 c.c. of agar, it developed two different types of colony—(*a*) thick, viscous colonies corresponding to those of the original strain, and (*b*) thin, transparent, non-viscous colonies.

Subcultures from the thin colonies reproduced on ordinary culture medium similar thin, non-viscous colonies; thus from the original growth a variant strain could be isolated differing in cultural characters from the original strain and remaining true to type even after repeated subculture. In all the other general characters the new strain corresponded to the original.

The original culture did not show variation in colony characters when plated on ordinary medium. It had been used as a type in certain serological investigations, and there could be no doubt as to its purity since the strain had been originally obtained after three successive platings and subculture from single colonies. An agglutinating serum had been prepared by immunisation of a rabbit.



Colonies on agar plate after 4 days at 37° C.

Subcultures from a thick viscous colony on the brilliant green agar plate replated on the same medium again yielded different types of colony, viz.: Type A — thin, non-viscous colonies as in the first experiment; Type B—small, thick, opaque, markedly viscous colonies; Type C—large, thick, opaque, non-viscous colonies; and also large opaque viscous colonies corresponding to those of the original strain. The different appearances of these colonies are shown in the photograph.

Primary subcultures from colonies A, B and C reproduced the respective colony characters; repeated subcultures remained true to type and there was

no tendency to reversion on subculture. On microscopic examination types B and C were more coccoid in shape, while A represented typical bacillary forms. By overstaining with Leishman's stain (ten minutes with the pure stain and thirty minutes with a 1 : 2 dilution in distilled water) a thick, somewhat irregular capsule was demonstrated in the case of B, while A and C were apparently non-capsulate. Hiss's method failed to show a distinct capsule. It was found that after three or four days' incubation, from some of the large, thick, viscous colonies representing the original strain, thin transparent borders were developed and appeared to correspond to the thin colony variant; subcultures from these borders reproduced thin colonies of type A, which remained true to type. It was also found later that subcultures from the large, thick, viscous colonies on the original brilliant green agar plate, replated on ordinary medium, likewise developed colonies from which the type A variant grew out in the form of a thin border. Thus from the original strain three different variants had been developed, as regards growth characters on solid culture medium; the biochemical reactions and other cultural characters of the original strain were reproduced in the new types, and it was shown how the variant A actually developed from the original type of colony.

Agglutination tests were carried out with the antiserum to the original strain, and striking differences in agglutinability were noted between the different types. The end-titre of the reaction with the original strain was 1 : 1600; type A was agglutinated by the same serum in dilutions up to 1 : 10,000; C displayed a lesser agglutinability than the original strain, the end-titre being 1 : 1000; in the case of B the end-titre was 1 : 3000 (Table I).

These results are of special interest as demonstrating variation in agglutinability associated with variation of other characters. On retesting these variants after several subcultures the agglutination results were found to be constant.

Similar observations were made with another *B. vesiculosus* strain to which an agglutinating serum had been prepared. The original strain grew in the form of large, white, opaque, non-viscous colonies. On brilliant green agar two types of colony were observed: (1) Those which represented apparently the original strain, and (2) thin transparent colonies resembling variant A of the first experiment; subcultures from these remained true to type as regards the new character, but did not differ in other general characters from the original strain. Agglutination tests yielded results which corresponded to those found in the first experiment; thus the thin colony variant again proved much more agglutinable than the original strain (Table II). Similar mutations as regards colony characters were observed with two other *B. coli* strains, and the variants remained true to type even after repeated subculture. It is to be noted that many *B. coli* strains grown on medium containing a slightly inhibitory concentration of brilliant green may develop thin colonies, but on subculture they revert to the original type.

TABLE I.

Agglutinating Serum to B. vesiculosus *Strain H.*

	Serum dilutions.										
	1 : 400.	1 : 800.	1 : 1000.	1 : 1200.	1 : 1600.	1 : 2000.	1 : 3000.	1 : 4000.	1 : 6000.	1 : 10,000.	1 : 20,000
Original strain	++++	+++	+++	+++	++	—	—	—	—	—	—
Variant A . .	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	—
„ C . .	+++	+++	+	—	—	—	—	—	—	—	—
„ B . .	++++	++++	++++	++++	+++	++	+	—	—	—	—

Reading after 4 hours at 37° C. Emulsions all of equal density.

TABLE II.

Agglutinating Serum to B. vesiculosus *Strain L.*

	Serum dilutions.										
	1 : 800.	1 : 1000.	1 : 2000.	1 : 3000.	1 : 4000.	1 : 6000.	1 : 8000.	1 : 10,000.	1 : 12,000.	1 : 15,000.	1 : 20,000
Original strain	+++	++	++	++	+	+	—	—	—	—	—
Variant . . .	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	—

Reading after 4 hours at 37° C. Emulsions of equal density.

The question, of course, arises as to whether variations in agglutinability might be dependent on morphological differences, *e. g.* the presence of a glutinous capsule. In the first experiments the most agglutinable variant was the A type (large, thin, non-viscous colonies), the least agglutinable the C type (large, thick, non-viscous colonies), while the original strain (large, thick, viscous colonies) and the B type (small, thick, viscous colonies) represented intermediate degrees of agglutinability. No conclusions can be drawn to show that agglutinability is influenced by the presence of a glutinous capsule. These observations are, however, of special interest in showing how the serological characters of a particular organism, interpreted by the usual methods, may vary under certain conditions.

REFERENCES.

1. Massini, R.—"Ueber einen in biologischer Beziehung interessanten Kolistamm (Bacterium coli mutabile). Ein Beitrag zur Variation bei Bakterien," *Arch f. Hyg.*, 1907, **61**, 250.
2. Penfold, W. J.—"Variability in the gas-forming power of intestinal bacteria," *Proc. R. Soc. Med.*, 1911, **4** (Part 3, Pathol. Sect.), 97.
3. *Idem.*—"Further experiments on variability in the gas-forming power of intestinal bacteria," *J. Hyg.*, 1912, **11**, 487.
4. Fletcher, W.—"Capsulate mucoid forms of paratyphoid and dysentery bacilli," *J. R. Army Med. Corps*, 1920, **34**, 219.